UNIVERSITY OF THESSALY
SCHOOL OF HEALTH SCIENCES

FACULTY OF VETERINARY SCIENCE
DEPARTMENT OF MICROBIOLOGY AND PARASITOLOGY

ECOLOGY AND MANAGEMENT OF EUROPEAN BROWN HARE SYNDROME IN MEDITERRANEAN ECOSYSTEMS

A thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Christos K. Sokos MSc, MSc

SUPERVISOR: Charalambos Billinis, Professor

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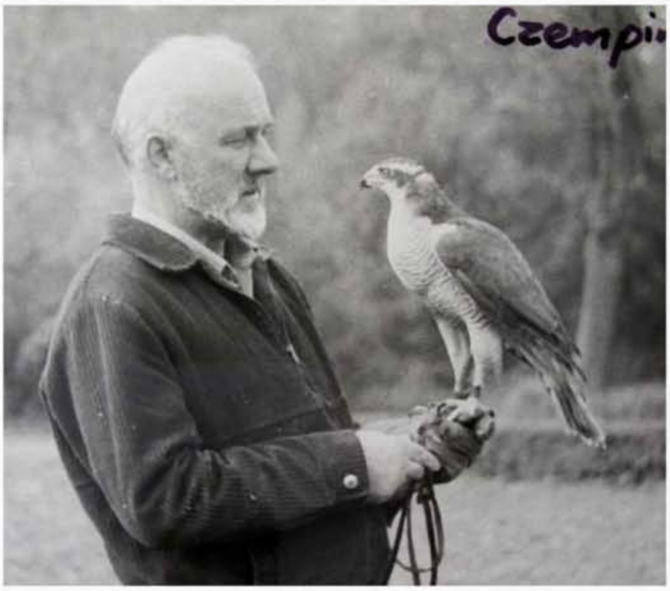
Dedicated to Prof. Zygmunt Pielowski
A pioneer hare researcher in Europe



Czempiń - Śp. Prof. Zygmunt Pielowski

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ABSTRACT

ECOLOGY AND MANAGEMENT OF EUROPEAN BROWN HARE SYNDROME IN MEDITERRANEAN ECOSYSTEMS

A Doctor of Philosophy dissertation by Christos K. Sokos MSc, MSc Wildlife Ecologist

Supervisor: Charalambos Billinis, Professor, Faculty of Veterinary Science

Many aspects of wildlife diseases, especially in Mediterranean ecosystems, including ecological parameters and management practices, remain uncertain. The European brown hare (*Lepus europaeus*) is a major wildlife species in Mediterranean Basin taking into account its importance as a prey for many carnivore species and as a huntable species. The recorded widespread mortality by European Brown Hare Syndrome (EBHS), indicates this viral disease as the most important disease for hares in Europe. This PhD thesis presents a series of studies conducted with the aim to investigate several aspects of the ecology and management of hare and EBHSV in Macedonia, Hellas.

Mediterranean ecosystems are characterized by a variety of ecotopes. The free access of Hellenic hunters in countryside gave the opportunity to study hare harvest and EBHSV prevalence in different ecotopes. During the study, 291 hares (*Lepus europaeus*) were collected by the hunters. The ecotope, where each hare was initially found by the dogs, was recorded and a population index was calculated. Moreover, the PCR was used for the detection of European Brown Hare Syndrome Virus (EBHSV). The greatest harvest in unit area was recorded in areas with mosaic of cereals and woody vegetation; in contrast the lowest harvest took place in forestlands. EBHSV had widespread distribution even in isolated areas and the prevalence was in an endemic stability of 17.8%. EBHSV has higher prevalence in Olive groves, followed by mixed cultivations and shrublands. Population index is related positively with EBHSV prevalence and harvest. Harvest is not related significantly with EBHSV prevalence. In a second step, a more detailed study was taken place with the contribution of multivariate statistics and Geographic Information Systems (GIS). It was found that prevalence increases in areas with higher hare abundance; closer to

paved road network and in lower altitudes. Secondly, a potential distribution map was constructed to show the relative risk of EBHSV in the study area.

Associations of EBHSV infection, sex, age, body condition and spleen mass were studied in 259 hares collected. Data showed a sex-biased prevalence with twice as many males infected with EBHSV than females, indicating a sexual dimorphism in disease exposure or susceptibility. EBHSV infection was not related to hare body condition and a higher body condition was found in males than in females. Adults had a higher ratio of infected animals than young but this difference was not found to be statistically significant. Adults also had higher spleen mass thus indicating past infections. An enlarged spleen was more frequently found in hares positive for EBHSV and a negative relation was found between spleen mass and body condition thus indicating energy consumption for immune defense investment.

Ecosystem disturbances, such as wildfires, are driving forces that determine ecological processes, including diseases. Species respond differentially in wildfires, having diverse post-fire population evolution. Hare relative abundance, age ratio, diet quality, body condition and diseases were determined after a wildfire in Kassandra Peninsula of Chalkidikis. The severe habitat disturbance by wildfire caused initially a reduction in hare abundance, although after one year and for four years hares were more abundant in burned area. Wildfire seems not to influence EBHSV prevalence between burned and unburned areas.

Nocturnal spotlight survey is a common census technique for many mammal species and is also used for samples collection. The influence of abiotic factors may be essential for the appropriate design of censuses; however this method has not been examined sufficiently in relation to abiotic factors such as weather conditions and the moon. We tested their influence in the case of hare. Wind speed and direction had an influence on hare counts with stronger winds resulted in fewer juvenile hares being observed, and more hares were counted when wind was blowing from the hare towards the vehicle. No significant effects were found by the other factors probably due to the intense relief and thick vegetation of the studied areas.

Hare release is a frequent management measure in some countries, and thousands of hares are released annually, mainly for hunting purposes. Survival rates in the wild are low for hares intensively reared in cages but are higher for hares reared extensively in enclosures and wild translocated hares. The benefits of the hare release practice are significantly lower than the action's implementation cost. Hare releases have not increased significantly the wild hare population or the hunting harvest in areas where the practice has been applied. The risk of genetic and evolutionary degradation and pathogen transmission is possible in wild populations.

The impact of EBHSV on hare populations appears to be highly variable. These studies have provided documentation on this variability and its parameters. Taking into account these ecological parameters of disease prevalence, the necessities on disease prevention are described more efficiently and the proposal of appropriate management measures becomes possible.

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Christos Sokos

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INTRODUCTION

The brown hare (*Lepus europaeus*) hereafter hare, spreads across most of Europe and has been introduced by human to other continents (Map 1). The hare is farmed for hunting and meat production and is an important species for hunting, especially in Europe (Pielowski 1976) and Mediterranean (Thomaides et al. 2007, Papaspyropoulos et al. 2014). However in some countries hare belong to a category of conservation concern (Smith et al. 2008) and in some areas hare has very low population densities (Sfougaris et al. 1999, Delibes-Mateos et al. 2009) with high conservation importance due to the increased genetic variability and geographical substructuring of populations (Kasapidis et al. 2005, Antoniou et al., 2013). Hare also has fundamental role in natural processes, either as prey for endangered predator species or by contributing to seed dispersal (Izhaki & Ne'eman 1997, Chapman and Flux 2008, Delibes-Mateos et al. 2009). Moreover, hares are reservoirs of important diseases (Wibbelt and Frölich 2005).

Map 1. Distribution range map of Lepus europaeus. In red the regions that the species introduced by human (IUCN Red List).

Hunting records of harvested hares can vary considerably from year to year on how favorable the conditions have been, but there is no evidence of cyclical population fluctuations (Tapper and Parsons, 1984). Marboutin and Peroux (1996) showed that harvests were synchronous between neighbouring districts in France showing that trends are at least locally synchronous. Harvest records also indicate a steady decline since the early 1960's in several European countries (Fig. 1). Several infectious diseases can occur in hares and may contribute in population decline (Lamargue et al. 1996, Wibbelt and Frölich 2005).

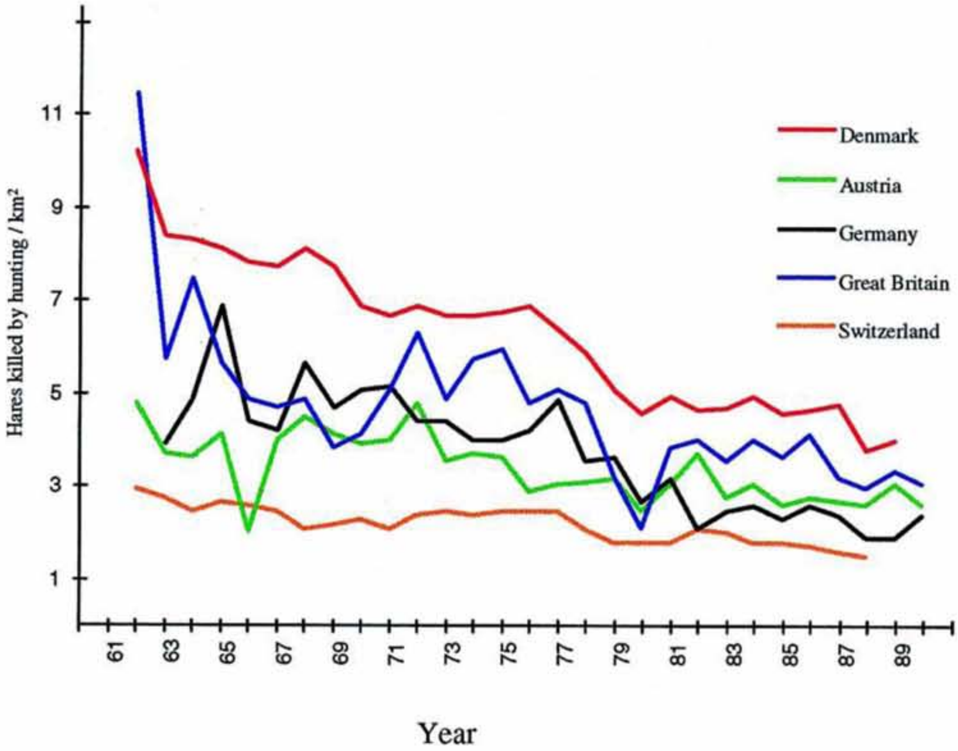

Fig. 1*. Trends in the numbers of Hares shot/km² during the 1960s, 1970s and 1980s in five European countries (Mary and Trouvilliez 1995).*

The impact level to hare population varies between different pathogens (Wibbelt and Frölich 2005). Lamarque et al. (1996) showed that >50% of hares found dead in France between 1986 and 1994 died from disease, principally European Brown Hare Syndrome (EBHS), Yersiniosis (Pseudotuberculosis), Pasteurellosis and Coccidiosis. Coccidiosis is enhanced in wet weather whereas EBHS tends to have epidemic years following high leveret production. Juveniles, after losing their protective maternally derived antibody, probably become susceptible to EBHS (Duff et al., 1997) with disease outbreaks killing most hares in September through January (Lamarque et al. 1996). Hares have also been identified as carriers of diseases that cause consequences to humans and domesticated animals as Brucellosis, Tularemia, Leptospirosis Toxoplasmosis and Neosporosis (Wibbelt and Frölich 2005, Treml et al., 2007, Bartova et al., 2010). Hare is in close contact with human through hunting and rearing

(Deutz et al. 2003, Treml et al. 2007) and livestock using pastures (Gortazar et al. 2007), thus may consist an important host species.

The epidemiological dimensions of many diseases are related to environmental factors and demographic parameters like age ratio and population density, diet and weather conditions (Wobeser 2006). The worms *Protostrongylus* sp. affecting the lung of the hare are dependent by the presence of gastropods as an intermediate host (Frolich et al. 2003). However, many aspects of hare diseases, especially in Mediterranean ecosystems, including ecological parameters and management practices, remain uncertain (Gortazar et al. 2006).

This thesis reports several studies conducted to investigate aspects of the ecology and management of EBHS in a variety of Mediterranean ecosystems in Macedonia, North Hellas. The reports on these studies are preceded by a literature review on hare infectious diseases, whereas the literature relevant to each of the specific research areas is reviewed in more depth in the introduction of each chapter.

In detail, the thesis is presented in the following chapters:

Chapter 1: Literature review about the agents causing the main infection diseases, to hare, including epidemiology in hare populations, ecology of diseases, management options and importance for human and livestock.

Chapter 2: Study on comparison between hare habitats, hare harvest and EBHSV prevalence.

Chapter 3: An in depth study on EBHSV prevalence distribution and ecological factors and management practices with the help of GIS.

Chapter 4: Study on pathogens and demographic and physiological parameters of hare populations.

Chapter 5: Study on the forest fires and the possible influences on hare population and EBHSV prevalence.

Chapter 6: Study on the influence of several factors, like weather and moonlight effects on hare population surveys and samples collection.

Chapter 7: Study on the influences of hare releases, including the prevention by pathogens.

Chapter 8: General discussion and conclusions of the results and implications for management and further research.

All samples used in this PhD thesis represent opportunistic samples that were collected for purposes other than the WildTech project. With regard to ethical considerations, all activities were performed strictly according to the European and Greek guidelines.

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1. IMPORTANCE AND MANAGEMENT OF HARE DISEASES

1.1 Brown hare infectious diseases and management

Wildlife infectious disease management

Wildlife disease management is a relatively new field in research and management and provides an opportunity to integrate ecological and veterinary knowledge (Henke et al. 2007). Disease in wildlife occurs at the interface between human medicine, veterinary medicine and wildlife ecology (Wobeser 2007). Wildlife infectious diseases have become increasingly important, as around 75% of emerging diseases are of wildlife origin (Billinis 2013). The increasing interest in wildlife diseases is attributed to: 1) the occurrence of zoonoses which have an important contribution to wildlife, 2) the recognition that wildlife serves as a reservoir of important diseases in domestic animals, 3) an increase in wild animal rearing and the risk of transmission of pathogens in wild populations through releases and translocations, and 4) intensification of conservation programs for species threatened with extinction or of economic importance (Wobeser 2002, Billinis 2013).

Initially, one would support that managing diseases in wild animals is impossible. According to Wobeser (2007), this thought is locked into the view that disease management involves only medication and treatment of sick animals. However, in 1933 Aldo Leopold (the father of wildlife science) stated that wild animals “are being doctored daily, for better or for worse, by gun and axe, and by fire and plow”, giving emphasis to environmental and population factors.

Wobeser (2007) refers that management can be attempted by manipulating three parameters: the agent, the host and the environment. The aims of management are: 1) prevention which involves the exclusion of pathogens, 2) control which involves the restriction of incidence and severity of existing diseases, and 3) eradication which involves total extirpation from an area.

Management of the causative agent includes: 1) preventing the introduction of agents into new areas, 2) the use of drugs, 3) reducing the agent in the external environment (carcass collection and egg destruction), and 4) reducing the vectors involved in pathogen transmission. Management of the host includes: 1) manipulation of animal distribution and dispersal, 2) selective removal of diseased animals, 3) reduction of population density (depopulation in a specific site or as barrier or in a large area), and 4) immunization. Management of the environment includes: 1) modifying abiotic and biotic factors, and 2) influencing human activities (Wobeser 2007, Delahay et al. 2009). Thus, managing diseases in wild animals is a possible task including options and should more included in future plans of responsible agencies.

Brown hare infectious diseases and management

Brown hare is a common wildlife species that often comes into contact with humans as it is a hunted and reared animal. The hare is a carrier of zoonotic diseases (Deutz et al. 2003, Treml et al. 2007) and in fact hunters have high seroprevalence against some zoonoses (Deutz et al. 2003, Jenzora et al. 2008). Hares are often bitten by hunting dogs and some hunters give uncooked internal hare organs to their dogs thus increasing the probability of infection and transmission of pathogens (Kirino et al. 2009). Hare use the same feeding places as farm animals (Juste et al. 2009), in some case preferably (Karmiris & Nastis 2007), and can also host their pathogens (Juste et al. 2009, Siembieda et al. 2011). Consequently, the presence of hare may be a problem for responsible agencies attempting to eradicate infectious diseases.

Additionally, hare is a common wildlife species with a broad worldwide distribution and management measures are applied to assist its population increase, including habitat improvement, releases and predator control (Reynolds et al. 2010). Hare has fundamental roles in natural processes as it is prey for threatened predator species (Chapman and Flux 2008, Delibes-Mateos et al. 2009) and is one of the most important sedentary quarry species for hunting economies (Papaspyropoulos et al. 2014).

About 30 pathogenic microorganisms have been identified in hares, and each of these has different implications for hare populations, humans and domestic animals (Wibbelt and Frölich 2005). Most of them are bacteria that cause diseases such as yersiniosis, pasteurellosis, tularemia, brucellosis, and borreliosis. European brown hare syndrome (EBHS) is the most important virus disease and causes mortality in hares. Regarding parasites, nematodes are commonly found, toxoplasmosis is an important zoonosis, while coccidia play an important role in mortality of juvenile hares (Lamarque et al. 1996). Although in most cases parasites can live within the organism without consequences, they can act secondarily and in synergy with other factors can increase mortality.

Sterba (1982) found that parasitic and infectious diseases were responsible for 25% and 30% of the deaths of 2269 brown hares in the Czech Republic, respectively. Other causes were dietary, toxicity and injury that were responsible for 25, 10 and 10% of the deaths, respectively. In Switzerland, infectious diseases had lead to the deaths of 15% of the dead hares found, and cases of pasteurellosis, brucellosis, pseudotuberculosis, tularaemia, listeriosis, and toxoplasmosis were diagnosed (Haerer et al. 2001).

The above parameters deem the full understanding of hare diseases essential. The aims of this chapter were therefore, firstly to evaluate the importance of the major infectious diseases occurring in hare by reviewing the current literature on the threats caused by hare pathogens to humans, hare populations and domestic animals. Secondly, preventive measures are described. The systematic literature review was performed according to Littell (2008). Research questions, bibliographic databases and websites were selected, as well as appropriate keywords to search the database of the Web of Science and Google Scholar. Following the recommendation of Tranfield et al. (2003) that searches should not be restricted to bibliographic databases, we also used Google to identify unpublished studies, conference proceedings and other relevant publications. Some additional publications were also found from reference lists. The following keywords were used for these searches: "*Lepus europaeus*", "diseases", "medicine", "pathogens", "host", "zoonotic", "domestic animals", "livestock", "management", "hunting dogs". In this review we dealt with publications from 1990 onwards.

Tularemia

Tularemia is caused by the bacterium *Francisella tularensis*, which is a highly infectious coccobacillus, frequently found in surface waters (Wibbelt and Frölich

2005, Esmaeili et al. 2014). Outbreaks of tularemia have been described in many countries and in some areas the disease is endemic (Petersen & Schriefer 2005, Esmaeili et al. 2014). Studies have indicated a higher prevalence in hunters and in individuals that had contact with hares (Petersen & Schriefer 2005, Jenzora et al. 2008, Schätzle and Schwenk 2008, Esmaeili et al. 2014). A study of 142 patients in Castille-Leon in northwestern Spain indicated that 97.2% of them had previous contact with hares; 83.8% had skinned hares and 13.3% had handled hare meat (Petersen & Schriefer 2005). *F. tularensis* subsp. *holarctica* was isolated from these patients and also from hares (Petersen & Schriefer 2005). Infection way of the local hares is not known, but the importation of infected hares from countries where tularemia is endemic is the most likely hypothesis (Pérez-Castrillón et al. 2001). Importation of infected hares is also referred by Lamarque et al. (1996) who found that most cases of tularemia in hares are reported during cold, wet seasons, especially during November and February. In November the disease appeared one to two months after the release of imported hares in France, whereas February is a month with high tick activity and ticks are reservoirs and vectors of disease (Lamarque et al. 1996).

Hare presence seems to aid the persistence of disease in endemic areas. In the province of Pavia in Italy there is an area that is endemic for tularemia and the disease has been found both in hares and humans (Magnino et al. 2011). Tularemia causes serious bacteraemia in hares and may thus be a major source of infection for blood sucking arthropods which are vectors of tularemia and whose excrement and carcasses may contaminate the environment (Treml et al. 2007). A relatively low percentage of tested hare sera found were positive to *F. tularensis* (Table 1). This occurs because mortality is often high and death occurs before the development of a detectable antibody response. Therefore, Magnino et al. (2011) note that direct methods of detecting tularemia in hares should be preferred to serological methods.

Pasteurellosis

Pasteurellosis is caused by the bacteria of the Pasteurellaceae family (Wibbelt and Frölich 2005, Iaria and Cascio 2007). From a public health perspective, pasteurellosis belongs to the list of infective agents that are potentially transmitted after animal contact, such as bites, scratches and licking of wounds (Iaria and Cascio 2007). In immunocompromised humans, *P. multocida* infection may cause septicemia, pneumonia, meningitis, endocarditis, and chronic abscesses characterized by extensive edema and fibrosis (Bisgaard 1993, Iaria and Cascio 2007, Guet-Revillet et al. 2013).

Pasteurellosis is a common disease in hare (Haerer et al. 2001, Wibbelt and Frölich 2005). The disease is usually peracute with septicaemia, haemorrhages and death occurring within 12 to 48 hours, or acute to chronic with fibrino-purulent pleuro-pneumonia and fibrinous pericarditis with death occurring after a few days or weeks (Kötsche and Gottschalk, 1990, Wibbelt and Frölich 2005). Sex and age do not influence the disease (Louzis et al. 1989, Lamarque et al. 1996). The disease has been diagnosed as the cause of death to 7–8.3% of hares found dead in the wild (Lamarque et al. 1996, Morner 1999). Cases of pasteurellosis in hares are reported throughout the year, with a slight increase occurring in spring (Lamarque et al. 1996).

Yersiniosis

Yersiniosis is a common zoonotic bacterial disease of hare. Human yersiniosis is primarily caused by *Yersinia enterocolitica* and less frequently by *Yersinia pseudotuberculosis.* Yersiniosis is an important hare disease (Sargent 1974, Haerer et al. 2001, Wibbelt and Frölich 2005) and common serotypes have been reported between human and hare infections of *Y. enterocolitica* and *Y. pseudotuberculosis* (Niskanen 2010). In hares, the disease is acute to chronic with dyspnoia and diarrhoea. Diseased hares show coordination disorders, weakness, weight loss and diarrhoea (Kötsche and Gottschalk 1990). Typical lesions are multifocal necroses within spleen, liver, intestine surrounded by macrophages, epithelioid cells and mesenteric lymph nodes (Dietz 1990, Wibbelt and Frölich 2005).

Most cases of yersiniosis in hares are reported during cold, wet seasons, and wet areas are prone to host the disease for longer (Lamarque et al. 1996, Wibbelt and Frölich 2005). Frölich et al. (2003), found antibodies against pathogenic *Yersinia* spp. in 55% of brown hares in Germany (Table 1). The disease has been diagnosed as the cause of death for 10.2–50% of hares found dead in the wild (Lamarque et al. 1996, Morner 1999, Wibbelt and Frölich 2005).

Swine Brucellosis

Brucellosis is a disease caused by bacteria of the genus *Brucella*, which are gram-negative and non-sporing (Wibbelt and Frölich 2005). Hare is host to swine (porcine) brucellosis (*Brucella suis*), mainly variety biovar 2 (Godfroid et al., 2005, Treml et al. 2007, Bekele et al. 2011). This biovar infects pigs and occasionally cattle (Godfroid et al. 2013), and is considered to have low pathogenesis in humans (Godfroid et al. 2005, Treml et al. 2007).

The role of hare in the transmission of swine brucellosis is considered of lesser importance than that of wildboar by some authors due to the no crossing and shorter dispersion and movements of hare (Godfroid et al. 2013). However, hare is more abundant in open farm areas and near human settlements (Smith et al. 2004) and may share more often the same feeding places with grazing pigs, where hare body fluids spread over soil and vegetation. Another way of transmission in domestic pigs is the feeding of pigs with offal obtained from hunted infected hares (Godfroid et al. 2013).

In hares the disease is usually chronic (Lamarque et al. 1996, Morner 1999, Wibbelt and Frölich 2005). Morner (1999) and Lamarque et al. (1996) did not find hares that had died from brucellosis in Sweden or France, respectively. A relatively low percentage of hare sera tested were found positive to *Brucella suis* (Table 1). Independence between the hare population density and cases of brucellosis in hares has been found in the Czech Republic, this indicates that brucellosis can persist even in low population densities of hares (Pikula et al. 2005).

Leptospirosis

Leptospirosis is caused by the spirochetes of *Leptospira* genus (Bharti et al. 2003) and has a diverse range of impacts to hosts from acute to chronic form of disease (Bharti et al. 2003). Rodents are the main hosts, however hare can host the disease and thus contribute to the maintenance of the pathogen in a specific area, and to human infection as hares are hunted and consumed (Deutz et al. 2003, Treml et al. 2007). Deutz et al. (2003) found leptospira antibodies in 10% of hunters and the authors refer that hare may contribute to infestation. In the Czech Republic, the main serotype found in hares was of *L. grippotyphosa* (95.04%) and secondly of *L. bulgarica* (4.96%) (Treml et al. 2003). Winkelmayer et al. (2005) found that seropositivity varied considerably between areas, and only antibodies against *L. interrogans* serovar Grippotyphosa were found. Leptospirosis does not cause mortality in hares (Lamarque et al. 1996, Morner 1999, Winkelmayer et al. 2005).

Table 1. Main brown hare infectious diseases and their impact on humans and domestic animals (high impact = ↑, medium impact = ↔, low impact = ↓, no impact = –) in Europe according to literature published after 1990. Impact increases with the reported frequency of outbreaks in humans and domestic animals. Hare is both a host and a vector of disease.

Disease	**Country**	**% positive for antibodies**	**% positive for isolation**	**Human cases in 2011**[1]	**Domestic animals**	**Source**
Tularemia				724[1]	↓	
	Germany	10.24				Dedek et al. (1990)
	Italy	0				Zanni et al. (1995)
	Germany	0				Frölich et al. (2003)
	Austria & Czech Republic	6				Winkelmayer et al. (2005)
	Czech Republic	6.5				Treml et al. (2007)
	Germany		1.1			Runge et al. (2011)
Pasteurellosis				?	↔	
Yersiniosis				7 041[1]	↔	
	Germany	16.2				Dedek et al. (1990)
	Germany	55				Frölich et al. (2003)
	Germany	89.6				Bartling et al. (2004)
Brucellosis				?	↑	
	Germany	0				Frölich et al. (2003)
	Austria & Czech Republic	3.54				Winkelmayer et al. (2005)
	Slovakia	0				Jurcik (2007)
	Czech Republic	1.6				Treml et al. (2007)
Leptospirosis				526[1]	↓	
	Germany	27				Dedek et al. (1990)
	Serbia	7.4				Trifunovic et al. (1991)
	Czech Republic	29				Zitek & Babička (2000)
	Czech Republic	15.89				Treml et al. (2003)
	Austria & Czech Republic	8.3				Winkelmayer et al. (2005)
	Czech Republic	7.5				Treml et al. (2007)

[1]The confirmed case rate of disease per 100.000 habitats in Europe according to the European Centre for Disease Prevention and Control, Annual Epidemiological Report 2013.

In conclusion, hare is infected by important infectious diseases – although the disease that has caused the most important population losses in Europe is EBHS (Lamarque et al. 1996, Wibbelt and Frölich 2005). Moreover, the above review gave some knowledge about hare management that can contribute to mitigation of hare diseases risks. Such measures are: 1) the careful handling of hares by hunters, 2) the restriction of translocations and hare releases in wild, and if a such action is necessary the examination of animals for pathogens, 3) the exclusion of hares by areas with susceptible farming animals, and 4) the control of population densities of hares.

1.2 European Brown Hare Syndrome

European brown hare syndrome (EBHS) is a hepatopathy of high mortality in brown hares and mountain hares (*Lepus timidus*). EBHS is caused by a calicivirus first described in Sweden in the early 1980s (Gavier-Widén and Mörner 1991). The viral character of the disease was demonstrated in 1989 (Lavazza and Vecchi 1989). However, lesions consistent with EBHS have been described from England since 1976 (Duff et al. 1994) and antibodies were found in hares archived since 1962 (Duff et al. 1997), although diseased hares were only recognized in the UK in 1989 (Duff et al. 1997). Today EBHS epidemics have been reported all over Europe and the infection has become endemic (Frölich and Lavazza 2008).

EBHS is caused by a non-enveloped, positive-strand RNA virus (EBHSV) of the genus *Lagovirus*, Caliciviridae family, with a diameter of about 28 to 30 nm (Nauwynck et al. 1993) or 32-35 nm (Frölich and Lavazza 2008). EBHSV and Rabbit Hemorrhagic Disease Virus (RHDV) both belong to genus *Lagovirus* and have significant similarities in their epidemiology, clinical signs, and pathology (Gavier-Widén and Mörner 1991). EBHS occurred before the emergence of RHD and in hare populations of similar areas to those affected by RHD in Europe. In addition, di Modugno and Nasti (1990) found that cross-infection of these viruses in the heterologous species was possible. Studies have found a 52-60% nucleotide homology between the viruses RHD and EBHS, indicating that they are distinct caliciviruses, although closely related to each other (Nowotny et al. 1997).

The origin of EBHSV is difficult to trace. It may have mutated from an avirulent calicivirus of Eurasian lagomorphs or by the introduction of a new virus avirulent in its native distribution, possibly from cottontail rabbits (*Sylvilagus* sp.) or hares introduced into Europe in the 1970s and 1980s (Frölich and Lavazza 2008).

There is evidence that EBHSV might have occurred in European hare populations many decades before pathological signs of EBHS were identified. A less pathogenic variant of EBHSV has been found in hares of Argentina, introduced there in the 19th century, consequently an ancestor of the present European EBHSV strain might have been apathogenic (Frölich et al. 2003). Moreover, Frölich et al. (2007) found indications for an apathogenic strain of the virus in Slovakia.

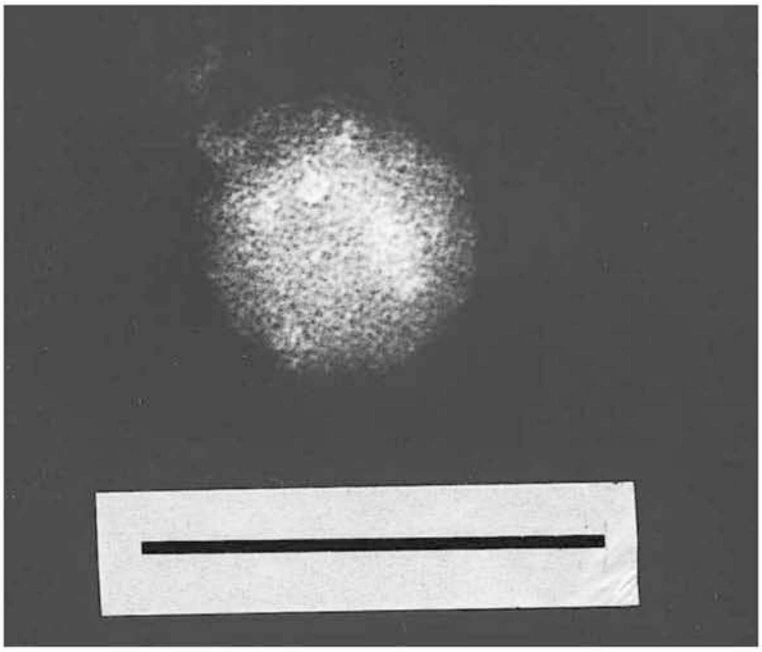

***Fig. 1.** Electron micrograph of a negatively stained calicivirus EBHSV. Bar = 100nm (from Frölich 2001).*

EBHSV isolates belong to one serotype and phylogenetic analysis of geographically different EBHSV strains revealed close overall homology (Frölich and Lavazza 2008). EBHSV strains are differentiated geographically and earlier strains survive longer than those of RHD viruses (Le Gall-Recule et al. 2006). The same authors suggest that new EBHS viruses have appeared and coexist with the previous ones thus increasing their genetic diversity. Moreover, Frölich et al. (2007) did not find a correlation between haplotype and development of EBHS antibodies in hare nor between haplotype and harboring of EBHSV infection in Slovakia. In contrast, Iakovakis et al. (2013) found the potential contribution of the MHC class II genotype of hare in differential susceptibility to EBHSV.

The pathology of EBHS

The infection is lethal in 35–60% of cases, and morbidity can reach 100% (Zanni et al. 1993, Zanni et al. 1995, Duff et al. 1994, Drews et al. 2011). The disease has not been observed in leverets younger than 40-50 days, however juveniles younger than 2–3 months may contract the infection but do not develop the disease (Zanni et al. 1993, Scicluna et al. 1994, Lavazza et al. 1999, Drews et al. 2011). Passive maternal immunity protection accounts for the lack of deaths in leverets (Drews et al. 2011). Haemagglutination inhibition antibodies against EBHS have been found in recently born hares in areas where the disease is endemic (Gavier-Widen and Morner 1991). Hares without immunity defense can die after few days or may finally recover (Nauwynck et al. 1993, Drews et al. 2011).

In farmed hares, higher mortality was found in ages between three to eleven months compared to older animals (Zanni et al. 1993, Drews et al. 2011). Anti-EBHSV titre was recorded as between 1:80 and 1:2560, which dropped to 1:10–1:160 nine months later. A titre of 1:10 is considered as being protective against the virus (Lavazza et al. 1996). Eskens et al. (2000) found that for animals up to one year of age, the frequency and amount of EBHSV antibodies were the same as for older animals.

On the basis of the morphology of the liver changes, three forms of the disease have been described, an acute, a subacute and a chronic form (Gavier-Widen 1994, Billinis et al. 2005a). The body weight distribution of hares with EBHS appeared to have lower values than healthy hares, indicating that the disease may cause weight loss. However, the lower weight of EBHS-affected hares may reflect the higher susceptibility of stressed animals (from other causes such as poor nutrition and predators) to infection with EBHSV (Gavier-Widen and Morner 1993). On the other hand, according to Duff et al. (1994), affected hares usually are in good physical condition.

In its acute form EBHS has a short clinical course, neurological anomalies, oedema and congestion of tracheal mucosa with foamy haemorrhagic contents, acute hepatitis, circulatory dysfunction, haemorrhages and decolouration in the inner organs and liver and spleen enlargement (Capucci et al. 1991, Poli et al. 1991, Duff et al. 1994, Billinis et al. 2005b). Affected hares have been observed as uncoordinated and attempting to run away but dying after paralysis within a few minutes. One reported behavior of infected animals was 'lack of fear' (Duff et al. 1994) and many carcasses were collected in atypical places for hares, such as close to houses and roads (Sostaric et al. 1991). Poli et al. (1991), describe a diminished escape distance, loss of balance and occasional incapacity to move. Epistaxis was a clinical finding in some of these animals. Behavioral alterations were also observed in hares maintained in captivity. On hare farms, affected animals died mainly within 1 to 2 days (Poli et al. 1991).

Animals exhibiting the chronic form of EBHS show generalized jaundice of the mucosa and submucosa, liver degeneration and necrosis (Frölich and Lavazza 2008).

Detection of the virus in this form of the disease may imply that these hares could play a role in the transmission of EBHS (Billinis et al. 2005a). Whereas other healthy hares may have antibodies but they lack the virus particles (Cammi et al. 2003, Drews et al. 2011). High titers of EBHSV-antigen have been detected through ELISA in both liver and spleen samples of hares (Frölich et al. 2001). In chronic forms of the disease, the virus may be complexed by antibodies and is no longer excreted, as also described for RHD (Barbieri et al. 1996).

Animals that survive after infection benefit from developed immunity, and hares with antibodies may be found throughout the year, though most mortality occurs from September until March (Poli et al. 1991, Duff et al. 1994, Scicluna et al. 1994, Duff et al. 1997, Cammi et al. 2003; Frölich and Lavazza 2008). However, EBHSV seropositive hares were found most frequently between April and September in Poland (Frölich et al. 1996). The survival of infected animals is probably higher from April to September than from October to February. This may be explained by the higher number of younger hares present in spring and summer populations that can overcome the disease (Frölich et al. 1996).

Population impact and ecology of EBHSV

The impact of the disease on hare populations seems to differ greatly depending on the area considered, the period of the year, the population density and the presence of anti-EBHSV antibodies. In some cases local European brown hare populations can be reduced significantly, particularly where it occurs as a local epidemic for the first time (Drews et al. 2011), but the disease is probably not the cause of major reductions in hare populations (Gavier-Widen and Morner, 1993; Frölich et al., 1996, Duff et al. 1997).

Löliger and Eskens (1991) refer that the impact on local hare populations can vary from 7-90% mortality in Germany. A decreased significance in the importance of EBHS was found for one hare population between 1994 and 1997 (Eskens et al. 2000). In northern Italy night hare counts revealed a decrease in the number of spotlighted hares during the winter period from 27-40% after unusually high morality due to EBHS in 1986, whereas in hare farms mortality ranged from 30-90% (Poli et al. 1991). However, in Britain years with high mortality incidents due to EBHS, were not bad years for hare harvests (Duff et al. 1997).

EBHS was widespread in both wild and captive hares in northern Italy where the disease is endemic, with 47.6% of samples being found positive for the virus and 95% of samples testing positive for EBHSV antibodies (Scicluna et al. 1994). Moreover, in another study, EBHS was diagnosed in 78.3% of dead or ill hares, but only in 6% of healthy hares killed during the hunting season (Cammi et al. 2003).

Antibody prevalence (seroprevalence) ranged from 36-90% and significant differences were detected between areas in Slovakia (Frölich et al. 2007) and France (Guitton et al. 2006). Variation in seroprevalence may depend on the population density and the presence of animals which have survived the EBHS virus and subsequently act as carriers and excretors (Duff et al. 1997, Lavazza et al. 1999, Paci et al. 2011).

When hare density is low (<8 hares/km^2), the virus transmission may be reduced and most juveniles remain seronegative. When hare density is higher (>8-15 hares/km^2), mortality can be reduced due to the infection of hares younger than 50 days old and thus hares can develop protective immunity (Lavazza et al. 1999). However, according to Guitton et al. (2006), seroprevalences in three populations with hare density >10 hares/km^2 was below 30% for young hares, indicating low protection. In two other populations located about 50km south of the previous three, seroprevalence was increased in the third study year without carcass findings. One explanation may be the circulation of an apathogenic EBHSV strain (Guitton et al. 2006). Moreover, different abiotic factors between areas may influence the disease. Cooke et al. (2002), found a higher prevalence of antibodies against RHDV virus in areas that receive an annual rainfall higher than 400-500 mm.

Frölich et al. (2001), refer that the low population density of brown hares in Switzerland may explain why EBHS was not reported to cause losses in Switzerland until 2000. In Germany significant differences in seroprevalence had no relation with population densities (Eskens et al. 2000). In contrast, Riedel et al. (1991) refer a higher percentage of infected hares by increasing bag size . In Britain 90% of dead hares were found in high hare density areas – with more than 8 hares/km^2 (Duff et al. 1997). In central Italy, a lower percentage of seropositive animals was correlated to low hare densities (usually in hunting areas), whereas the seroprevalence was higher in areas with high and medium densities (usually in refuges) (Cammi et al. 2003, Paci et al. 2011).

Cammi et al. (2003), refer that the carcasses of hares that had died from EBHS were found more frequently in hunting areas than in refuges. This may be a consequence of higher presence of hunters in these areas, who observe and record the dead animals. However, a higher percentage of seronegative animals was detected in hunting areas by Cammi et al. (2003) and Paci et al. (2011) and this percentage increased with the distance from refuges (Paci et al. 2011).

Relatively little is known about the transmission of the disease. According to Frölich and Lavazza (2008): "Humans, insects, and birds can act as vectors but no reservoir hosts have been identified yet. Infection via ingested vegetation is also likely, with virus being secreted and excreted, and also being spread in the droppings of predators that have consumed infected hares. EBHSV is highly robust, resisting acid of pH3, and may remain infectious for 3-4 months in the field". Similarly, RHDV is also tolerant of broad ranges of temperature. Infected liver samples did not lose infectivity after storage at –5°C for 413 days, at –20°C for 560 days, or at –70°C for 4.5 years (Xu 1991). RHDV also resists heating to 50°C for 60 minutes (Xu 1991). RHDV dried on cloth survived at room temperature (approximately 20°C) for 105 days, although there was some evidence of reduced infectivity (Smid et al. 1991). These studies indicate the ability of EBHSV to survive in outdoor conditions facilitating the transmission.

EBHSV has also been found on islands, which has raised the question about the role of birds in the transmission the virus (Syrjälä et al. 2005). Transportation and vehicles have been also incriminated for the spread of the virus (Chasey 1994). In a previous study, the introduction of RHDV into the UK was attributed to passive virus transmission through vehicle traffic (Chasey 1994). Moreover, flies and other insects may have an important role in transmission, as found for RHDV cases (Asgari et al. 1998, McColl et al. 2002).

Hare management and EBHS

Measures to mitigate EBHS impacts in wild populations are usually not taken in hare management practices. However, official directives in some countries state that infected or diseased hares should not be released into wild. EBHS was not reported to cause losses in Switzerland until 2000, and one explanation was the prohibition of importation of hares for release for more than 10 years (Frölich et al. 2001). In Hellas, twelve of twenty hares which had mtDNA haplotypes matching those found in imported animals were positive for EBHSV, whereas 33 indigenous hares were all negative for EBHSV. Three virus sequences found in three imported and released hares were related to the northern–central European EBHSV strains. Therefore, alien strains of EBHSV were introduced with released hares, possibly resulting in negative impacts on indigenous populations that have not yet evolved resistance to these new virus strains, thus the importation of hares has now been prohibited in Hellas (Spyrou et al. 2013).

Other protective measures for wild populations refer to population density. Higher densities will show reduced mortality due to the development of protective immunity as a consequence of previous infection at the age of less than two to three months and the development of protective immunity (Lavazza et al. 1999, Cammi et al. 2003, Paci et al. 2011). Hare density increase is a common aim in hare management by responsible agencies and measures are taken for habitat improvement, predator control, hunting regulation, etc.. Therefore the stabilization of populations at higher numbers may protect from and reduces EBHS impacts.

Human activities are not usually taken also into account, although hunting practices cause an increased presence of people and dogs within natural hare habitats and thus may facilitate transmission of the virus. Natal dispersal rates of hares are higher in hunting areas than in non-hunting areas (Bray et al. 2007), and hunting may increase hare movements (Avril et al. 2014) – factors that may increase the probabilities of infection.

Dogs, either hunting, shepherd or feral, may transmit the virus after consuming contaminated hare carcasses. One example of RHDV transmission was described by Morisse et al. (1991): Reunion Island imports frozen rabbit meat from China. Following a cyclone in 1989, the breakdown of refrigeration units caused the dispersion of large numbers of rabbit carcasses in the surrounding area. Following this event, farmed rabbit colonies on the island became contaminated after dogs had contact with the Chinese rabbit carcasses. Moreover, infective RHDV was also found in faeces of dogs which had been fed with infected rabbit livers (Simon et al. 1994).

EBHS is also a problem in hare farms (Scicluna et al. 1994, Santilli et al. 2004, Drews et al. 2011). Poli et al. (1991), found that hare farm size did not influence the mortality rate by EBHS. In the case of RHD, the affected farms were typically small

(Löliger and Eskens 1991) and it was suggested that small farms may use green feed from local fields which was potentially contaminated by the virus from wild rabbits, whereas large farms prefer to use industrial feed (Schlüter and Schirrmeier 1991, Löliger and Eskens 1991). Santilli et al. (2004), propose the emptying of enclosures with the release of young hares before November when the incidence of disease increases due to higher population density after the breeding season, more favorable weather conditions for the virus, and to the offspring losing their protective antibodies.

Drews et al. (2011), describe the precautionary measures taken in a farm during an infection with cleaning and disinfection of the cages and vaccination of young animals. The effectiveness of the cleaning and disinfection measures were proven by the zero titre of the offspring born after the outbreak until the time of vaccination in December 2009. A good immune response to the vaccine was monitored after the vaccination. The second vaccination did not result in higher titres compared to the first, but did increase the duration of immunity. All the vaccinated hares exhibited a titre of at least 1:10, which is protective (Lavazza et al. 1996), whereas the majority of hares showed titres of up to 1:320 after the second vaccination. The wide range of titres observed between the animals might be attributed to individual differences in immune response. Drews et al. (2011), refer that because animals born in the future will also lose their passively acquired antibodies after weaning, they will vaccinate young hares when they are older than two months, the age when the young animals become susceptible to EBHS.

1.3 Shortcomings in the literature and research aims

This review of the literature on hare diseases and especially on EBHS has brought several omissions to light. Although the clinical, pathological and genetic aspects of EBHS are efficiently documented, there is a deficit of studies on the ecology and management of diseases in hare populations. Prevalence of EBHSV and its likely interrelationships with biotic and abiotic factors require more intensive investigation.

Firstly, although the variables seroprevalence and impact of the disease on hare populations (comparison between areas and epochs) is commented on in several studies, a comparison between different ecotopes, hunting harvest and EBHSV prevalence has not taken place. Moreover, spatial aspects of the EBHSV prevalence with the use of Geographic Information Systems (GIS) and the development of spatial risk models have not yet used. GIS is a very helpful tool and thus the influence of environmental factors can be examined more efficiently.

Secondly, EBHSV prevalence and the association with the demographic and physiology factors of hares is limited, although many studies reveal that sex, age, body condition and immunity differences between individuals may influence the proneness to becoming infected.

Thirdly, the survey methods for population estimation and samples collection for diseases monitoring should properly designed. However, the influence of several factors, like weather and moonlight effects, is generally overlooked in hare studies.

Fourthly, although the outbreaks of EBHS and seroprevalence to EBHSV often have been described, there has been little attention given to the causes of these outbreaks or to changes of prevalence after an environmental change. For example, habitat disturbances like wildfires are frequent happen in Mediterranean and their influence on the EBHSV prevalence has not been examined.

Finally, hare releases have been practiced for more than a half century in Europe, without taking into account the risks of diseases, although serious for the transmission of many important infectious diseases. The management and ecological issues arising from this practice are very important, however, have not been examined under an integrated study.

The purpose of my research project was to advance understanding of the ecology and management of EBHSV in Mediterranean ecosystems aiming in above shortcomings.

The high variability of biotic and abiotic parameters makes the Mediterranean region suitable for this investigation and in area that EBHS causes mortality to hares (Billinis et al. 2005b). The overall body of work was founded on the belief that better understanding of the wildlife diseases is essential for appropriate and optimal employment of management.

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2 THE EBHSV PREVALENCE AND HARE HARVEST AND POPULATION IN DIFFERENT ECOTOPES

Abstract

Mediterranean ecosystems are characterized by a variety of ecotopes. The free access of hunters in countryside gave the opportunity to study hare harvest and EBHS prevalence in different ecotopes. During the study, 291 hares (*Lepus europaeus*) were collected by the hunters. The ecotope, where each hare was initially found by the dogs, was recorded. Moreover, the PCR was used for the detection of European Brown Hare Syndrome Virus (EBHSV) in harvested individuals. It was found that the greatest harvest per unit area was recorded in areas with Mosaic of cereals and woody vegetation; on the contrary the lowest harvest took place in Forestlands. EBHSV has higher prevalence in Olive groves, followed by Mixed cultivations and Shrublands. Hare population was related with the harvest and EBHSV prevalence, although harvest and EBHSV prevalence were not related. The classification of ecotopes according to the hunting harvest and disease prevalence contributes to a better management of harvest, habitat improvement and disease control.

2.1 Introduction

Among the services of ecosystems is hunting (Papaspyropoulos et al. 2012). In Hellas it has been found that hare hunting has serious socio-economic benefits (Sokos et al. 2003, Papaspyropoulos et al. 2014). The hare is the main indigenous quarry species for which the 20% of hunting excursions of about 200,000 Hellenes hunters takes place (Thomaidis et al. 2011). A typical harehunter does 39 hunting excursions per year (Sokos et al. 2003, Platis and Skordas 2006). Considering that the number of harehunters in Hellas is 70,000 (30% of all hunters), it is estimated that 2.73 million hunting excursions take place annually for hare hunting in the country. The average hunter harvest is one hare per six trips, or one hare per three trips by a group of two hare hunters (Thomaidis et al. 2011), meaning that the number of harvested hares is estimated to 455,000 annually by harehunters. Several thousand hares are harvested by other non hare hunters, therefore estimated that about 500,000 hares are harvested annually in Hellas.

Additionally, there is a high added value in economy due to hare hunting; for example, in the accommodation sector, or the hunting accessories sector (Papaspyropoulos et al., 2012). The harehunters of Thessaloniki characterize their ιactivity irreplaceable because of the multifaceted benefits that are offered to them and spend an average of 39 days per year for hunting and 30 days for the training of hare dogs (Sokos et al. 2003). Consequently the commercial consumption by this activity is estimated on average 1,658 euros per year per harehunter (Sokos et al. 2003). Considering that the number of harehunters is 70,000, and then the commercial consumption is estimated at 116.06 million euros per year. The harehunters argue that a better management is needed to improve the quality of harehunting and declare 'willingness to pay' (Sokos et al. 2003). However, the existing responsible agencies do not respond adequately to the needs for populations increase and sustainable management (Birtsas et al. 2009) and this may is a reason for the reduction hunting licenses issued in the last 25 years in Greece (Tsachalidis 2003).

Hunting records of harvested hares can vary from year to year depending on how favorable the weather and habitat conditions have been, but there is no evidence of cyclical population fluctuations (Tapper and Parsons 1984). Marboutin and Peroux (1996) showed that harvests were synchronous between neighbouring districts in France showing that trends are at least locally synchronous. Harvest records also indicate a steady hare population decline since the early 1960's in several European countries (Mary and Trouvilliez 1995). Several infectious diseases can occur in hares and may contribute to population and harvest decline (Lamargue et al. 1996, Wibbelt and Frölich 2005). The disease with the highest impact on hare populations is the European Brown Hare Syndrome (EBHS), which also causes mortality to hares in Hellas since 1980s (Leontidis et al. 1999, Billinis et al. 2005).

Mediterranean Basin is characterized by a high diversity of ecotopes in nearby areas (Sokos et al. 2013); moreover the Hellenic hunters have the right to hunt freely everywhere in the level of a prefecture or a large geographic region without the permission of landowner. These facts allow and gave the opportunity for examining the hare harvest and EBHS prevalence in different ecotopes. Consequently, aim of the present study was to investigate the relationships between hare harvest, ecotypes and EBHSV prevalence.

2.2 Materials and methods

Study area and ecotopes classification

The study was conducted oin the Prefectures of Chalkidiki and Thessaloniki, Macedonia, Greece, in an area of 6,601 km^2. The study area is located at the Mediterranean Basin, a region with dry hot summers and mild winters: average temperature is between 23°C and 34°C in summer and between 4°C and 19°C in winter.

The study was conducted during three consecutive hunting season (September 15th to January 10^{th}) of the years2010-2013.. Hares were harvested by volunteer hunters which were legally licensed. The authors declare that this study is based on legal hunting methods according to the Hellenic Hunting Law (200148/2833/06-08-2010, Decision of the Minister of Agriculture). Initially, about 100 harehunters evenly spaced in two prefectures were informed about the study and a specially designed questionnaire was given.

Hunters noted the municipality in which they hunted the hare and pointed the site where initially they found the hare on a detailed color map. Using CORINE (Corine Land Cover 2006 http://www.eea.europa.eu/data-and-maps/data/clc-2006-vector-data-version) and Google Earth, the following seven ecotopes were distinguished according to the habitat needs of the hare, the characteristics of vegetation and land uses:

1) Forestlands: forestlands mixed with herbaceous vegetation openings, but without the presence of crops.

2) Shrublands: shrublands mixed with herbaceous vegetation openings, but without the presence of crops.

3) Olive groves: olive groves in mixed with forestland or shrubland vegetation.

4) Mosaic: mosaic of cereals and secondarily other non-irrigated crops (olive groves, vineyards) with forestland or shrubland vegetation (Mosaic).

5) Cereals: cereals and secondarily other non-irrigated crops (olive groves, vineyards).

6) Mixed cultivation: irrigated crops (cotton, maize) and non-irrigated crops and few patches of forest vegetation.

7) Irrigated crops: irrigated crops in lowlands.

Each hare hunted was connected to one of above habitat type after evaluating the area in 400 meters radius around the point that they were found. The radius of 400 meters was selected as it simulates the home range of the species (Reitz and Leonard 1994, Bertolino et al. 2013). Moreover, we calculated the area of the above seven habitats using the CORINE Land Cover program for each prefecture.

Population index of hares

An index of the relative abundance of hares was estimated by the methods of faeces counts and drive censuses. Additionally a questionnaire was answered by the hare hunters that provided the samples. More than one method were used for the estimation of hare abundance due to the fact that the increased relief, the dense vegetation and the extended study area may cause biases while also the cost of a labor intensive method in this extended region is prohibitive (Langbein *et al.* 1999). To minimize biases all the counts were performed by the author.

Initially, hunters were asked to evaluate the hare population in places that they collected the hares using a scoring scale 0-5. Then, the area was divided in 16 sub-areas according to the habitat, the hunter's evaluation, the landscape features, the barriers for hare movements and the hare samples collection.

In each sub-area eight belt transects for faeces counting were placed along the length of the unpaved roads near the places that the hares were collected. Belt transects were located every 600-800 m from each other along the road, after the random selection of the starting belt transect (Calvete *et al.* 2004). In each belt transect four sections of 100 m length were installed with 0.5 m width each (Fa *et al.* 1999; Calvete *et al.* 2004; Murphy & Bowman 2007). Two sections were located in each side of the road on a constant distance of 30 m from the road and two more sections were located on a constant distance of 50 m from the road. In cases where obstacles were encountered (logs, dense shrubs, steep slope), we went around until the 100 meters be completed. Consequently, faeces were counted in a total distance of 400 meters in each belt transect.

The sections were at a distance of 30 to 50 meters from the road so as not to be influenced by some negative factors, e.g. disturbance from cars and livestock. This method is considered to be more effective than setting plots (Litvaitis et al. 1985, Langbein et al. 1999) because it covers a larger area and thus, it is more likely to identify hare faeces in an area with low population density, and many zero values, which complicate the statistical analysis, are avoided. Faeces counting took place during the September and October of 2011 and totally 51.2 km were walked.

The faeces's counting was followed by the drive censuses in four representative sub-areas which presented different hare abundance as it was previously estimated by the faeces counting. In each sub-area five belts of about 50 ha (100m×5000m) were selected randomly but extensively wooded belts were excluded (Langbein *et al.* 1999). The hare counting was conducted by flushing with the help of experienced hare hunters and dogs while also the existence of hares indicated by dogs was taken into account (Dahlgren et al. 2012). In order to avoid double-counting, the hares found, were recorded in a map (Gutzwiller 1990).

RNA extraction and PCR amplification

Hares were harvested and selected organs were collected (liver, eye, etc.) for investigating hare diseases (see for details Chapter 3.).

Statistical analyses

The importance of each ecotope for hare harvest was calculated as the ratio of the percentage of hares hunted in each ecotope to the percentage of ecotope area in prefecture excluding lakes and human infrastructures. Similarly, we calculated the population and prevalence of EBHSV in each ecotope, correlated the pairs of three variables (harvest, population and EBHSV) in order to find out if there is a relationship between them. We used the Pearson correlation coefficient (Fowler et al, 2013). We also computed the harvest rate per unit EBHSV (HpE) prevalence and compared it between Thessaloniki and Chalkidiki. We also test if there is a significant difference in this variable between the different geographic regions. Given that we had only five common ecotopes between the two areas, we used the non parametric test Mann-Whitney to test for differences of HpE between the two areas. This test compares the distribution of the dependent variable (HpE) in the levels of the independent (area) (Gray and Kinnear, 2012). We used the statistical software SPSS 22.0 for the statistical analysis and we set the significance level at 5%.

2.3 Results

In total 56 hare hunters participated in the study,34 hunters in Thessaloniki and 22 in Chalkidiki. In total they collected 291 hares (172 hares were harvested in Thessaloniki and 119 in Chalkidiki (Table 1). In Thessaloniki the ecotope with the larger area is the Mosaic of woody vegetation and cereals (30.15%), while in Chalkidiki the Forestlands (28.09%). In Thessaloniki the ecotope of Olive groves and in Chalkidiki the ecotope of Irrigated crops are very restricted, and hares were not harvested (Table 1).

Table 1. Proportion of each ecotope area (%), hares harvested, unit EBHSV prevalence and the index of mean population abundance (hares/km²).

Ecotores and their land cover	% (1)	Thessaloniki						Ecot. % (1)	Chalkidiki					
		Hares harv.	Hares % (2)	(2)/(1)	Unit (2)/(1)	Unit EBHSV prev.	Pop. hares/ km²		Hares harv.	Hares % (2)	(2)/(1)	Unit (2)/(1)	Unit EBHSV prev.	Pop. hares/ km²
Forestl.	15.54	8	4.65	0.299	0.059	0.145	2.8	28.09	5	4.2	0.15	0.024	0.114	3.8
Shrubl.	17.94	32	18.6	1.037	0.205	0.228	4.4	24.94	16	13.44	0.54	0.085	0.179	5.2
Oliv. gr.	0.44	0	-	-	-	-	-	11.75	27	22.69	1.93	0.305	0.280	7.3
Mosaic	30.15	91	52.9	1.754	0.347	0.135	3.8	22.94	60	50.42	2.2	0.348	0.106	5.4
Cereals	12.4	16	9.3	0.750	0.148	0.169	3.1	6.63	6	5.04	0.76	0.120	0.133	4.1
Mixed cultiv.	12.97	16	9.3	0.717	0.142	0.239	3.2	5.65	5	4.2	0.74	0.117	0.187	3.4
Irrig. crop	10.5	9	5.23	0.496	0.098	0.081	2.5	0	-	-	-	-	-	-
Total	100	172	100	5.054	1	1	3.3	100	119	100	6.32	1	1	5.5

Taking into consideration the area of each ecotope in each prefecture, the highest number of harvested hares was recorded in the ecotope of Mosaic in both prefectures, followed by Olive groves in Chalkidiki and Shrublands in Thessaloniki. The Forestlands and lowland farmlands have the lower number for harvested hares. The index of mean population abundance is higher in Shrublands and Mosaic in Thessaloniki, whereas in Chalkidiki is in Olive groves folloed by Mosaic and Shrublands (Figure 1).

RT-PCR revealed 17.8% prevalence of EBHSV infection in hares older than 50 days old. EBHSV has the highest prevalence in Olive groves of Chalkidiki, followed by Mixed cultivations and Shrublands, whereas the lower is in Irrigated crops and in Mosaic.

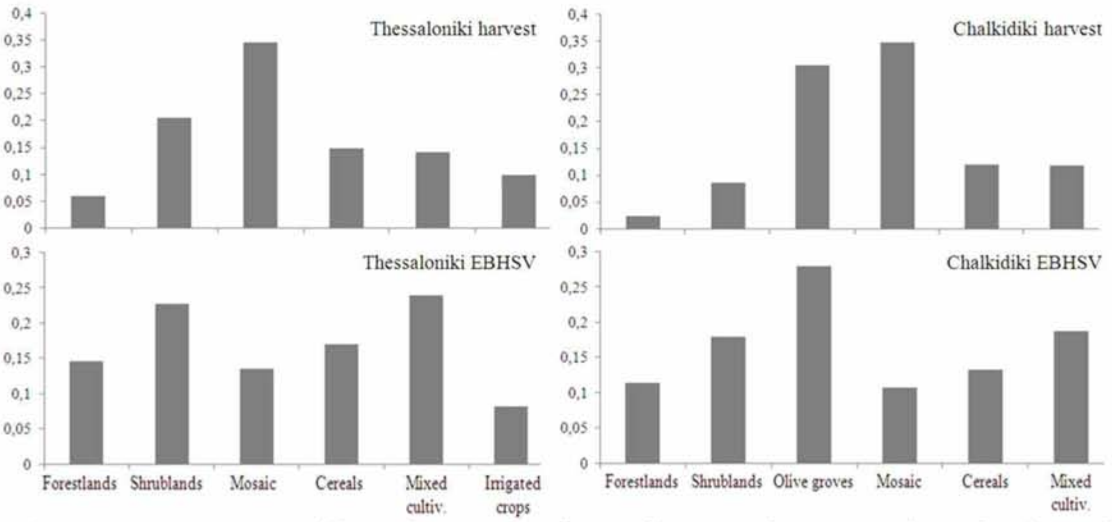

Figure 1. *Unit rates of hare harvest and EBHSV prevalence in Thessaloniki and Chalkidiki in corresponding ecotopes.*

Bivariate correlation analysis showed that the hare harvest is correlated to the population index (r=0.711, P=0.01), although is not correlated to the prevalence of EBHSV (r=0.205, P=0.522). The prevalence of EBHSV is correlated to the population index (r=0.619, P=0.032), with more infected hares found in ecotopes with higher population index (Figure 2). No significant difference was also found for the variable HpE. The Mann Whitney U Test was U=12.0 and the P value was 0.917> 0.05. Thus, the geographic region seems not to play role in the hare harvest under the EBHSV prevalence. In Mosaic, where the highest harvest was found, the EBHSV prevelance is lower. However, in Olive groves of Chalkidiki where the harvest is high, EBHSV prevelance is also high.

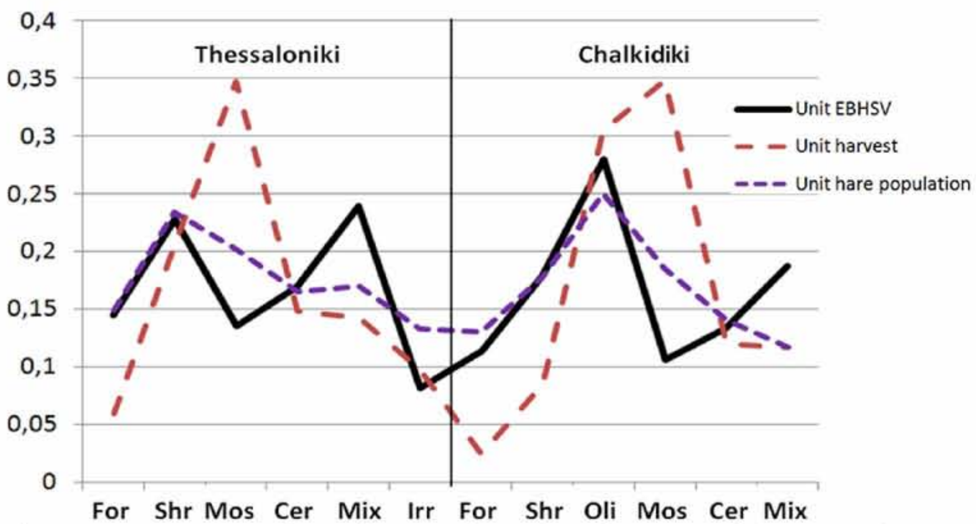

Figure 2. *Unit rates of EBHSV prevalence hare harvest and hare population, separately in Thessaloniki and Chalkidiki in corresponding ecotopes (For: Forestland, Shr: Shrubland, Mos: Mosaic, Cer: Cereals, Mix: Mixed cultivation, Irr: Irrigated crops).*

2.4 Discussion

The hare harvest and EBHSV prevalence are not the same among the different types of ecotopes. The harvest is 14.5 times lower in Forestlands in comparison with Mosaic and EBHSV prevalence threefold in Olive groves in comparison with Mosaic. Similarly, in Slovakia (Frölich et al. 2007) and France (Guitton et al. 2006), the reported seroprevalence differed significantly among the areas of the same country.

The relation of harvest and EBHSV prevalence is not significant. Harvest can be dependent by hunter behavior, the relief and vegetation, and also the population. Many factors contribute to population limitation of hares (Smith et al. 2005), and all these can confound the impact of EBHS on population and harvest. In this study there is no evidence that the EBHS causes a serious population reduction and harvest.

The highest hare harvest was recorded in Olive groves and Mosaic obviously due to the habitat suitability resulting in higher hare population. These ecotopes have the higher diversity and the most complex vegetation structure as forestlands, shrublands and cultivations coexist in the landscape. The hare is benefited by the combination of woody (trees and shrubs) and herbaceous vegetation, as the first provides cover and the second food (Sfougaris and Gkarabeli 2006, Zaccaroni et al. 2009, Bertolino et al. 2013). The influence of woody vegetation depends on the size of area covered and its distribution. Sites with patches of woody vegetation that are surrounded by low herbaceous vegetation have positive effect on hare population, but if the woody cover is high this is negative for the hare numbers (Schneider and Maar 1997, Vaughan et al. 2003). A reason can be a higher presence of predators and also a higher risk of predation especially in areas with trees (Bresiski and Chlewski 1976, Panek and Kamieniarz 1999, Torre and Diaz 2004, Paci et al. 2007, Tapia et al. 2008).

Our data indicated that Forestlands (mainly oak and pine forests) are connected to low harvest. The harvest is more lower than the population of hares in forests, especially in forests without openings as it has been also found by Sfougaris and Gkarabeli (2006), thus more reasons could be the difficulty of harvest by hunters due to the thick vegetation, the difficulty in flushing hares by dogs and also because forested areas are more remote and the access is difficult especially in winter period. It is characteristic that only one hare was hunted in forest from 1 November up to 10 January. On the contrary in lowlands the number of harvested hares is doubled after the 1st of November, possibly due to harehunters' trend not to travel far from home during the winter months (Sokos et al. 2003).

Except for the woody vegetation, the cultivation of openings seems to be also important. The harvest was higher in areas where openings are cultivated. Obviously, the cultivated openings, mainly from cereals or olive groves, may offer a diet of higher quality compared to herbaceous vegetation of Shrublands. Retzepis et al. (2005) found that hares harvested in sites with cereals had a better body condition compared to rangelands. Moreover, the olive groves are irrigated during the dry period in Chalkidiki, consequently herbaceous vegetation around the trees is maintained green during this season providing food of higher quality.

In Shrublands of Thessaloniki a higher harvest was found in comparison with Chalkidiki. This can be attributed to the fact that in most of Shrublands of Thessaloniki there are more openings, thus contributing to a higher hare density. A second reason may be the easier harvest of hares by hunters who are able to flush more easily the hares with their dogs and can follow easier the escape routes of hares.

In cultivated ecotopes (cereals, mixed and irrigated crops) the harvest is lower, possibly because of the lower population due to the absence of woody cover, but also because of the application of intensive agricultural practices (Vaughan et al. 2003, Sfougaris and Gkarabeli 2006). Similarly, Sfougaris and Gkarabeli (2006) found that the density of hares was extremely low in flat cultivated areas of Central Hellas. In this study area, hare densities in cultivated areas are not extremely low, but about 40% lower in comparison with ecotopes of Mosaic and Olive groves.

In conclusion, the hunting harvest, hare abundance and EBHS prevalence are differed among the seven ecotopes. Harvest and EBHS prevalence are not correlated significantly, probably due to the influence by other factors. This knowledge should be taken into consideration in the assessment of different ecotopes as hunting areas, but also in the application of management measures. Therefore it should be given lower priority in remote forested areas in the frame of planning of habitat improvements and other measures, due to their lower harvest value. Similarly, in lowland cultivated areas with intensive agricultural practices the habitat improvement may have high cost in comparison with the benefits and thus it is not recommended. Taking into account the EBHS, ecotopes with high prevalence should not be preferred in translocation programs.

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3. SPATIAL DISTRIBUTION OF EBHS VIRUS IN MEDITERRANEAN ECOSYSTEMS AND HARE MANAGEMENT

Abstract

1. The interaction between wildlife population and pathogens, along with environmental factors, leads to diverse prevalence of infection/disease and mortality rates, making thus difficult the risk assessment and the interpretation of epidemiological data. Understanding of the spatial distribution of pathogens facilitates the disease risk assessment and it is also important in managing. Relatively little are known about the spatial distribution of viruses that infect wild mammals in relation to environmental factors, especially in heterogeneous Mediterranean landscapes with intense human presence.

2. Here, multivariate statistics and GIS analysis methods were applied to estimate spatial patterns of biotic and abiotic factors and human activities as disease determinants of European Brown Hare Syndrome virus (EBHSV) prevalence in European brown hare (*Lepus europaeus* Pallas, 1778) in ecosystems of Macedonia, North Hellas (Greece). Environmental layers were created using the ArcGIS10.1 Geographic Information System (GIS) software (ESRI, Redlands, CA). Hare population abundance was estimated using faeces counts and belt drive censuses. Hares were sampled and a RT-PCR was performed to detect EBHSV.

3. The study showed that the disease had widespread distribution even in isolated areas and that during the two years of sampling it was in an endemic stability with 17.6% prevalence. However, prevalence increases in areas with higher hare abundance, closer to paved road network and in lower altitudes. In a next step, a potential distribution map was constructed to show the relative risk of EBHSV in the study area.

4. This study demonstrates that hare abundance and landscape influences spatial distribution of EBHSV, a finding that should be taken into account in future epidemiological studies. The implications for species management are discussed.

3.1 Introduction

The importance of pathogens as limiting factor of wildlife populations is becoming increasingly recognized, as there are multiple examples of pathogens being implicated in the decline or extinction of wildlife species. The inclusion of diseases in wildlife management is a relatively novel perspective giving new opportunities in mitigation of risks for populations (Delahay, Smith, & Hutchings 2009, Thirgood 2009, Joseph et al. 2013). Lagovirus, an emerging genus of Caliciviridae which includes the European brown hare syndrome virus (EBHSV) of hares, the Rabbit Hemorrhagic Disease Virus (RHDV) of rabbits and the RHDV related virus (RHDV2) of both rabbits and hares, cause acute and lethal hepatitis and are considered to be important diseases for the lagomorphs (Frölich and Lavazza 2008, Puggioni et al. 2013).

EBHSV, a highly contagious and fatal disease that affects wild and farmed hares of the species *Lepus europaeus* and *Lepus timidus*, is transmitted directly or indirectly by fecal, oral and respiratory routes. It has been reported that humans, insects, and vertebrates can act as vectors. However no reservoir hosts have been yet identified. The infection via consumed vegetation followed by virus secretion, excretion and spread in the droppings of predators that have consumed infected hares, has been also suggested (Frölich and Lavazza 2008). The adult animals are more susceptible whereas the disease has not been observed in leverets younger than 40-50 days. EBHS is lethal in 35–60% of infected hares and morbidity can reach 100% (Zanni et al. 1993, Zanni et al. 1995, Duff et al., 1994, Drews et al. 2011).

The impact of the disease on the hare populations varies, depending on the area considered and the time after the arrival of disease (Frölich and Lavazza 2008). According to Lavazza et al. (1999), when hare density is low (<8 hares/km^2), the virus transmission is reduced and most juveniles remain seronegative. When hare density is high (about 15 hares /km^2), mortality may be reduced due to the development of protective immunity as a consequence of previous infection in the age of less than two to three months and the development of protective immunity. In Central Italy, low seroprevalence was correlated to low hare density whereas the seroprevalence was higher in areas with high and medium population densitiy (Cammi et al. 2003, Paci et al. 2011). However, in Germany, no relation was reported between seroprevalence rate and population density (Eskens et al. 2000). In Britain, 90% of dead hares were found in high hare density areas-with more than 8 hares/km² (Duff et al. 1997). The controversial results from the studies mentioned above indicate a gap of knowledge about the determinants of the disease including other factors that may influence the infection prevalence except of the population density. These can be of an intrinsic nature such as characteristics of the hares or EBHSV or extrinsic such as environmental influences or human activities (Pfeiffer 2010). The extrinsic determinants of EBHS and the spatial distribution of EBHSV are still poorly studied.

The investigation of the spatial distribution of EBHSV is a special challenge due to difficulties such as the sample collection in wide areas, the counting of hare populations and the description of the environmental factors and human activities (Wobeser 1994, Ostfeld et al. 2005, Clements and Pfeiffer, 2009). Geographic Information Systems (GIS) have contributed to these studies and to the development of spatial risk models for infectious diseases. GIS is a very helpful tool for the creation, the management and the analysis of spatial data in order to associate the data obtained with epidemiological indicators including the infection prevalence and thereby to understand the disease ecology (Yilma and Malone, 1998, Clements and Pfeiffer, 2009). This knowledge will enable the disease prediction and management (Ostfeld et al. 2005, Bennema et al. 2009).

Therefore the aim of this study was to understand the EBHSV – habitat relationships and to predict the potential distribution of the virus in different although nearby ecosystems. The high variability of biotic and abiotic parameters makes the Mediterranean region suitable for this investigation. Specifically, the study has focused to correlate the EBHSV with the following: (1) biotic parameters, such as density of hare population, presence of livestock, and vegetation, (2) abiotic parameters, such as topographic and climatic conditions, and (3) human activities such as road network and hunting management (Hunting Prohibited Areas and the Hunting Dogs Training Areas).

3.2 Materials and Methods

Study area

The study was conducted in Macedonia, in Prefectures of Chalkidiki and Thessaloniki and in the island of Thasos, in a total area of 6,981 km². In region at

1999-2003 many dead hares were found (Billinis et al. 2005) and EBHS seems to was in an epidemic outbreak in. The last seven years about 1-2 hares are found dead by EBHS every year.

This region has high variety of ecosystems: lakes, rivers, salt marshes, irrigated and non-irrigated agricultural areas, maquis, broadleaved and conifer forests (**Fig. 1**). In the region there are barriers for hare movements like sea and closed fence highway roads. The altitudes ranging from sea level to 1140 m above sea level, the climate is Mediterranean with hot and dry summers: average temperature is between 23°C and 34°C during summer; and between 4°C to 19°C during the winter. Winters are mild near the sea and colder in inland and mountains, with mean minimum temperature of the coldest month ranging from below 0°C to 3°C. There are 37 Hunting Prohibited Areas (refuges) and 44 Hunting Dogs Training Areas in the region where dog training is permitted outside the hunting period,in spring and summer. There are also small hare farms in the region, the two of which use cage-reared hares and the other three are enclosures of about one hectare. The last ten years no hare release took place in the study area according to the archive of Hunting Federation of Macedonia & Thrace, due to the ineffectiveness of such a strategy in the increase of wild population, the genetic introgression and the risk of pathogen transmission (Spyrou at al. 2013). However, allopatric EBHSV strains have been found in the southern part of Chalkidiki Prefecture (Spyrou at al. 2013).

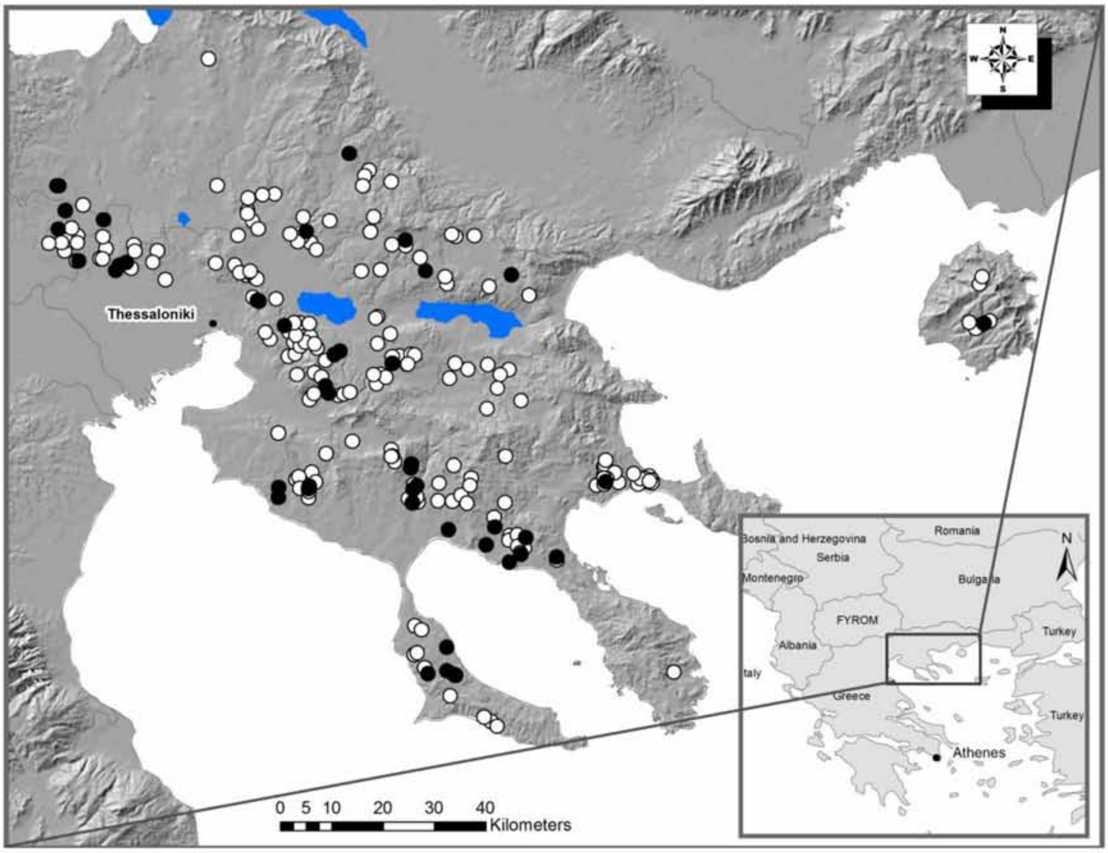

Fig. 1. *Location of the study area. The black bullets indicate the RT-PCR positive hares to EBHSV, whereas white bullets indicate the RT-PCR negative hares.*

Hares collection, RNA extraction, PCR amplification

No active capture, killing and sampling of wild animals specifically for this study took place. The majority of hares were taken by volunteer's hunters and few were found dead by vehicle collisions and predators. Initially about 100 hare-hunters were informed about the sampling collection in meetings and events of hunting organizations in the study area. A pre-sample of 98 hares showed prevalence of 16.3%. Consequently 206 hares can be an adequate sample size in order to estimate the disease prevalence in a large population at the 95% confidence interval (Pfeiffer 2010). Hares were collected during the hunting period (15/9-10/1), which is also a period of high incidence of disease (Scicluna et al. 1994). Hares were collected for two hunting periods: 162 hares for 2010-11 and 88 hares for 2011-12. Data on hare samples during the study were located in the field using handheld Global Positioning System (GPS) units or located by means in Google Earth software (http://www.google.com/earth/) by the samplers and Game wardens. A diagnostic post-mortem examination was conducted on all hares. The necropsy was performed according to the necropsy guide by Feldman and Seely (1988). Tissue samples from liver and spleen were obtained and stored at -80 ^{0}C until they were analysed.

Total RNA was extracted from all liver samples using the PureLink™ RNA Mini kit (Invitrogen Corporation, Carlsbad California, USA) according to the manufacturer's instructions.

RT was performed using reagents supplied with the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen Corporation, Carlsbad California, USA). A preliminary mixture containing 2 μl of RNA extraction product, 1 μl of dNTP mix (10 mM), 5 μl of Random hexamers (50 ng/μl) and 2 μl of DEPC-treated water was incubated for 5 minutes at 65°C and it was then placed on ice for at least 1 minute. Subsequently, 9 μl of a 2X reaction mix containing 2 μl of 10X RT buffer, 4 μl $MgCl_2$ (25 mM), 2 μl DTT (0.1 M) and 1 μl RNaseOUT™ (40 U/μl) were added to each RNA/primer mixture. After incubation in room temperature (~25°C) for 2 min, 1 μl SuperScript™ II RT (50 U/μl) was added to each tube followed by incubation in room temperature (~25°C) for 10 min. Reverse transcription reactions were carried out at 42°C for 50 minutes and they were terminated by heating at 70°C for 15 min. After chilling on ice, 1 μl RNase H (2 U/μl) was added in each reaction mixture followed by incubation at 37°C for 20 min. The reactions were stored at -20°C pending PCR.

PCR amplification was carried out in a final volume of 20 μl reaction mixture containing 2 μl of the cDNA reaction, 2 μl of 10X PCR Buffer, 2 μl dNTP mix (2,5 mM), 0,8 μl $MgCl_2$ (50 mM), 0,2 μl Taq DNA Polymerase (5 U/μl), 20 pmoles of each primer and up to 20 μl DEPC-treated water. Primers HEF 5'-CCGTCCAGCATTCGTCCTGTCAC-3' (nt1 795–1817) and HEB 5'-CATCACCAGTCCTCCGCACCAC-3' were selected from the VP60 gene of EBHSV (nucleotides 6423 to 6687 on the complete genome sequence of EBHSV GD, accession no. Z69620). The cycling conditions were initial denaturation at 94°C for 9min followed by 35 cycles consisting of denaturation at 94 ◦C for 45 sec, annealing at 60 ◦C for 45 sec and extension at 72 ◦C for 1 min, followed by a final extension at 72 ◦C for 7 min. Amplification products were subjected to electrophoresis in 2% agarose gel stained with ethidium bromide (0.5 μg/ml) and visualized under ultraviolet light. Product sizes were determined with reference to a 100-bp molecular-weight ladder. The expected RT-PCR product size was 265bp.

Abundance of hares

The relative abundance of hares was estimated by the methods of faeces counts and drive censuses. Additionally a questionnaire was answered by the hare hunters that provided the samples. More than one method were used for the estimation of hare abundance due to the fact that the increased relief, the dense vegetation and the extended study area may cause biases while also the cost of a labor intensive method in this extended region is prohibitive (Langbein *et al.* 1999). To minimize biases all the counts were performed by the author.

Initially, hunters were asked to evaluate the hare population in places that they collected the hares using a scoring scale 0-5. Then, the area was divided in 16 sub-areas according to the habitat, the hunter's evaluation, the landscape features, the barriers for hare movements and the hare samples collection.

In each sub-area eight belt transects for faeces counting were placed along the length of the unpaved roads near the places that the hares were collected. Belt transects were located every 600-800 m from each other along the road, after the random selection of the starting belt transect (Calvete *et al.* 2004). In each belt transect four sections of 100 m length were installed with 0.5 m width each (Fa *et al.* 1999; Calvete *et al.* 2004; Murphy & Bowman 2007). Two sections were located in each side of the road on a constant distance of 30 m from the road and two more sections were located on a constant distance of 50 m from the road. In cases where obstacles were encountered (logs, dense shrubs, steep slope), we went around until the 100 meters be completed. Consequently, faeces were counted in a total distance of 400 meters in each belt transect.

The sections were at a distance of 30 to 50 meters from the road so as not to be influenced by some negative factors, e.g. disturbance from cars and livestock. This method is considered to be more effective than setting plots (Litvaitis et al. 1985, Langbein et al. 1999) because it covers a larger area and thus, it is more likely to identify hare faeces in an area with low population density, and many zero values, which complicate the statistical analysis, are avoided. Faeces counting took place during the September and October of 2011 and totally 51.2 km were walked.

The faeces's counting was followed by the drive censuses in four representative sub-areas which presented different hare abundance as it was previously estimated by the faeces counting. In each sub-area five belts of about 50 ha (100m×5000m) were selected randomly but extensively wooded belts were excluded (Langbein *et al.* 1999). The hare counting was conducted by flushing with the help of experienced hare hunters and dogs while also the existence of hares indicated by dogs was taken into account (Dahlgren et al. 2012). In order to avoid double-counting, the hares found, were recorded in a map (Gutzwiller 1990).

Environmental variables

Environmental variables for this study were required from three main database categories: climate, elevation and land cover data. WorldClim version 1.4 climate data was obtained from the WorldClim website (http://www.worldclim.org). WorldClim is a set of global climate layers (climate grids) with a spatial resolution of 1 square kilometer. Landscape variables including altitude, aspect and slope were extracted from a digital elevation model (DEM) with a spatial resolution of 1 square kilometer (http://srtm.csi.cgiar.org/Index.asp). Land uses were derived from the Corine Land Cover 2000 database (European Environment Agency – EEA, http://www.eea.europa.eu/data-and-maps). Village and vegetation corrections were digitized from 2007 and 2009 color orthophotos that were available through Web Mapping Service (WMS) (http://gis.ktimanet.gr).

To create environmental layers for the analysis (Table 1), ArcGIS 10.1 GIS software was used (ESRI, Redlands, CA, USA). GIS layers were created to represent factors such as the locations of towns and villages, distance to the nearest village, distance from water presence, distance from livestock units, hare farms etc. For many of the above parameters, neighborhood statistics for radii of 100, 200, 500 and 1000 m were calculated to determine which spatial scale affects the presence of cases most strongly. A topographic position index classified the landscape according both topographic position (i.e. ridge top, valley bottom, mid-slope, etc.) and landform category (i.e. steep narrow canyons, gentle valleys, plains, open slopes, mesas, etc.). These data sets were converted to a common projection, map extent and resolution prior to use in the modeling program. Hares were also classified into seven habitat types according to the main land use in a radius of 500m: forests, shrublands, olive groves in mix with shrublands, cereals in mix with shrublands and forests, cereals, irrigated crops in mix with non-irrigated crops, irrigated crops.

Table 1. Main environmental variables used in the analysis

Variable	Value	Source
Hare population density	continuous	Field Work
Slope (degrees) at 100-, 200-, 500- and 1000-m radii	X, SD, min, max	DEM
Topographic position index (4 classes)	binary	DEM
Altitude	continuous	DEM
Aspect	N,W,S,E	DEM
Distance from nearest village	continuous	ArcGIS-WMS
Distance from livestock units, road network, wildlife refuges, hare farms	continuous	GEoDatabase
Distance from water	continuous	ArcGIS-DEM
Habitat types (7 classes: forests, cultivations, etc.)	categorical	ArcGIS-Corine LC (EEA)
19 Climatic variables (Temperature 11 indices, Precipitation 8 indices)	continuous	World Clim Database
NDVI (Normalized Difference Vegetation Index) 12 indices	continuous	World Clim Database
Human population density	continuous	GEoDatabase

Statistical analyses

The data set used, consisted of 47 variables including one dependent (bivariate variable Positive/Negative, concerning the existence of EBHS) and 46 independent variables (IVs)

The independent variables were relevant to categorical variables (hares population, habitat, if the hare was found in a hunting dog training area), landscape variables (altitude, slope, aspect, distances from forest roads etc.), and vegetation and climatic continuous variables (temperature, precipitation, NDVI for every month etc.).

The hares' sample was in total 250 specimens. In previous studies, it has been reported that in order to draw some reliable conclusions, at least 10 cases per IV must exist for a regression analysis. Therefore, the 46 IVs had to be reduced into a smaller set of variable, with the least loss of variance. Firstly, some descriptive statistics were extracted for the basic IVs. Then, the multivariate method Exploratory Factor

Analysis (EFA) was used (Hair et al. 2010) to achieve this reduction. As there were many independent variables to analyze, being strongly correlated, it was deemed appropriate to work with a smaller set of new variables-factors. These new variables, the factors produced from this method, account for the greatest variance of the initial set of variables and are uncorrelated among them. EFA was applied with the principal components as the factor extraction method. Factor rotation was done using the Varimax with Kaizer normalization method. The optimum number of factors was extracted using the Kaizer criterion combined with the Variance explained criterion. Therefore, the factors should have an eigenvalue above one in order to be selected, and the total variance accounted for by the factors should be around 80%. Factor scores were extracted with the Anderson-Rubin method, which produces scores with mean values of 0, standard deviations of 1, and uncorrelated (Hair et al. 2010). This procedure was performed three times: i) to reduce the 12 NDVI variables, ii) the Temperature variables, and iii) the Precipitation variables.

Our aim was to include the new factors with the rest of the IVs into a Binary Logistic Regression. Logistic regression is a category membership prediction technique which carries few theoretic assumptions for the variables used in the analysis. It can also use categorical variables as IV (Gray and Kinnear, 2012). However, due to the presence of multicollinearity and after several attempts to model the DV with these IVs, a simple independent samples t-test was applied to control for differences of the variables and factors between the hares with the EBHS and the hares without it. Levene's test was used to check the homogeneity of variances of each variable, taking into consideration the additional p-value available when the variances were not equal.

The variables with significant differences between positive and non-positive hares were then used again in the Binary Logistic Regression. Our aim was to determine if positive hares were influenced significantly by some independent variables compared to the non-positive hares. The analysis was performed using IBM SPSS 20.0 (Gray and Kinnear, 2012) with significance level 5%.

EBHSV predicted distribution

Mahalanobis distances D were calculated with ArcGIS software, based on the values of the three statistical significant variables, allowing us to identify potential distribution areas for EBHSV. Prior to running the Mahalanobis distance we chose the three variables based on multivariate statistics of our database. We calculated a multivariate statistic, Mahalanobis distance, to develop a model for brown hares and predict EBHSV potential distribution. Mahalanobis distances are calculated as: $D^2 = (x - m)^T C^{-1}(x - m)$

where:

D^2= Mahalanobis distance

X=vector data of environmental parameters at the locations sampled

m= is the mean vector of environmental parameters

C^{-1}= is the inverse of the variance-covariance matrix, calculated from the positive cases.

T= Indicates vector should be transposed

D^2 is a statistical measure of dissimilarity between the given point and the mean for all locations, and is expressed as a distance. We calculated D^2 with Arc GIS program based on the values of three map layers (road network, population density and altitude) and ‘‘ideal’’ values of those variables associated with positive EBHSV locations occurred in brown hares to identify suitable areas for BHSV potential distribution and occurrence (Beier and Brost 2010, Jenness et al. 2013). The analysis was performed using ARC GIS 10.1.

3.3 Results

Descriptive statistics

Hares younger than 50 days old were negative to EBHSV and they were excluded from the analysis because the disease has not been observed at this age in previous reports. A total of 250 samples were finally included in our study and 44 of them turned out positive for EBHSV (17.6%). The percentage of EBHSV RT-PCR positive hares was 17.9% during 2010-2011 and 17% during 2011-2012. No statistically significant difference was found between the two time periods (Chi-Sq=0.045, P=0.831). Moreover, there was no statistically significant difference between dry-warm period (15/9-31/10) and wet-cold period (1/11-10/1) (Chi-Sq=0.003, P=0.955).

RT-PCR positive hares were found in all habitat types without difference in prevalence rates ($F_{1,249}$=0.92, P=0.337). In our study area, the hare population density ranged from 1–9 hares/km² with a mean density of 4.56 hares/km². The hare population abundance was found to be an important factor for EBHSV circulation (Fig. 2) as we detected more EBHSV positive hares in areas with higher hare abundance ($F_{1,249}$=6.3, P=0.013). In dog training areas 7 out of 40 hares examined were positive. No statistically significant difference was found in the prevalence rate between the training areas and the areas where training was prohibited (Chi-Sq=0.00, P=0.984). Moreover, no statistically significant difference was found in the prevalence rate in hare farms between a zone of 5km and a zone of 5 to 10km (Chi-Sq=0.001, P=0.972).

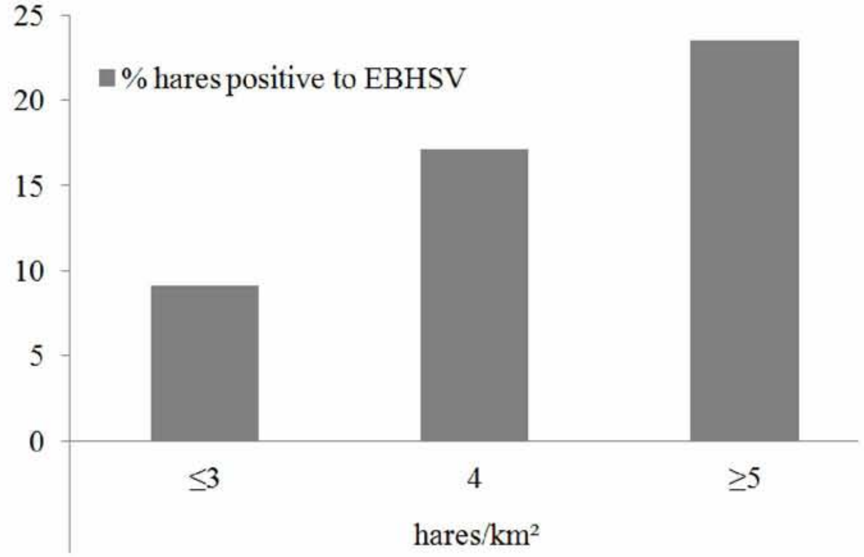

Fig. 2 *Hares positive to EBHSV (%) in relation with hares abundance.*

Table 2 shows some descriptive statistics of basic independent variables. The first column block shows the mean and standard deviation for the total sample, the second for the positive hares and the third for the negative hares. Independent variables have a high variability. For example the altitude ranges from 6m to 878m a.s.l., the distance from paved road network ranges from 90m to 4321m and the distance from water presence ranges from 0m to 4149m. The t-test showed that two continuous variables were statistically significant different between the two groups of hares. These variables were the distance from paved road network (t-test = 3.657, p = 0.000) and the altitude (t-test = -2.249, p = 0.042). Hares collected in distance closer to 350m from paved road network had a twice prevalence compared to hares collected in longer distances (27.8% vs. 14.5%), whereas hares collected in distance above 1500m had a prevalence of only 3.6%. Hares collected above the 200m altitude had 32% decreased prevalence (21% vs. 14.3%).

Table 2. Descriptive statistics, mean and Standard Deviation (SD) of the main continuous independent variables.

	Total		Positive		Negative	
	Mean	SD	Mean	SD	Mean	SD
Altitude	271.5	193.0	232.2	133.6	288.6	198.6
Distance from forest roads	263	280.0	216.9	212.4	272.8	292.0
Distance from paved roads	997.5	715.1	720.6	504.5	1,056.6	740.1
Distance from water presence	1,006.9	912.7	832.8	586.1	1,044.1	965.3
Distance from " livestock units	1,063.7	703.3	1,123.6	811.3	1,050.9	679.5
Distance from villages	2,648.3	1,264.4	2,451.0	1,330.7	2,690.5	1,249.1
Aspect	179.9	95.1	174.8	93.0	181.0	95.7
Slope	12.8	32.5	8.2	25.1	13.8	33.9
Human population	64.8	96.2	66.6	109.7	64.4	93.3
Annual Mean Temperature	15.0	3.0	15.1	2.4	15.0	3.1
Annual Precipitation	464.0	56.1	460.3	40.6	464.7	59.0
Distance from wildlife refuges	4,756.0	3,954.6	5,272.7	4,395.4	4,645.6	3,856.7

Multivariate statistics

Table 3 shows the number of factors-new variables extracted by applying three times the PCA. They correspond to the 12 NDVI variables, the 11 temperature variables and the 8 precipitation variables. The results on Table 3 show that there are now only 6 factors-variables compared to 31 previously, and there is a very small loss on the total variance that is explained from these factors. Thus, the application of PCA reduced significantly the available dataset.

Table 3. Results from PCA analysis

Variable category	Initial number of variables	Factors extracted	Eigenvalues after rotation	Variance explained	Total variance explained
NDVI	12	2	8.602	71.68	96.75
			3.009	25.07	
Temperature	11	2	7.473	67.94	93.80
			2.845	25.86	
Precipitation	8	2	4.242	53.03	87,18
			2.732	34.15	

The six new climatic and vegetation variables together with the independent variables presented in Tables 2 (excluding annual precipitation and annual mean temperature), and the four variables: dog training area, habitat type, hare population abundance and Topographic Position Index were combined in Binary Logistic

Regression. The rule of thumb of at least 10 cases per predictor variable was taken into account. Therefore 20 independent variables were used for 250 hares (dependent binary variable). However, several attempts showed that no significant model was reached. Therefore, the t-test was performed as which showed that from the new climatic and vegetation variables only Temperature Factor 2 (t-test = -2.030, p = 0.043) was statistically significant. The last variable-factor was related to the initial variables "Mean Temperature of the coldest quarter" and "Minimum Temperature of the coldest month".

After the iterations of the Binary Logistic Regression procedure by including the three significant continuous variables and the three variables: dog training area, habitat type, hare population abundance and their interactions, the most efficient model was the model shown in Table 4. The above model had a Nagelkerke R square of 0.584 (medium to high effect). Hares' abundance seems to play the more significant role, and it appears that when the abundance of hares is high there is a greater possibility for a hare to be positive. Prevalence seems to be influenced by the paved roads and altitude, and the positive hares are closer to roads and in lower altitudes. Habitat type, dog training zone and Temperature Factor 2 did not have a significant influence.

Table 4. *Variables' coefficients in the logistic regression*

	B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B)	
							Lower	Upper
Hare abundance	0.675	0.280	5.809	1	0.016	1.964	1.134	3.401
Paved road network distance	-0.004	0.001	11.386	1	0.001	0.996	0.993	0.998
Altittude	-0.001	0.000	6.096	1	0.04	0.999	0.999	1.000

A prediction map of EBHSV was constructed on the basis of factors identified as contributing to the presence of EBHSV which are population density, distance from paved road network and altitude (Fig. 3). Map displays the potential geographic distribution of EBHSV, predicted by GIS and Mahalanobis distance. The areas presenting high prevelance rate were recognized; most of them were concentrated in the south-west of Chalkidiki Prefecture and in central of Thessaloniki Prefecture. The areas of Koufalia and Ormylia present the higher presence of infection.

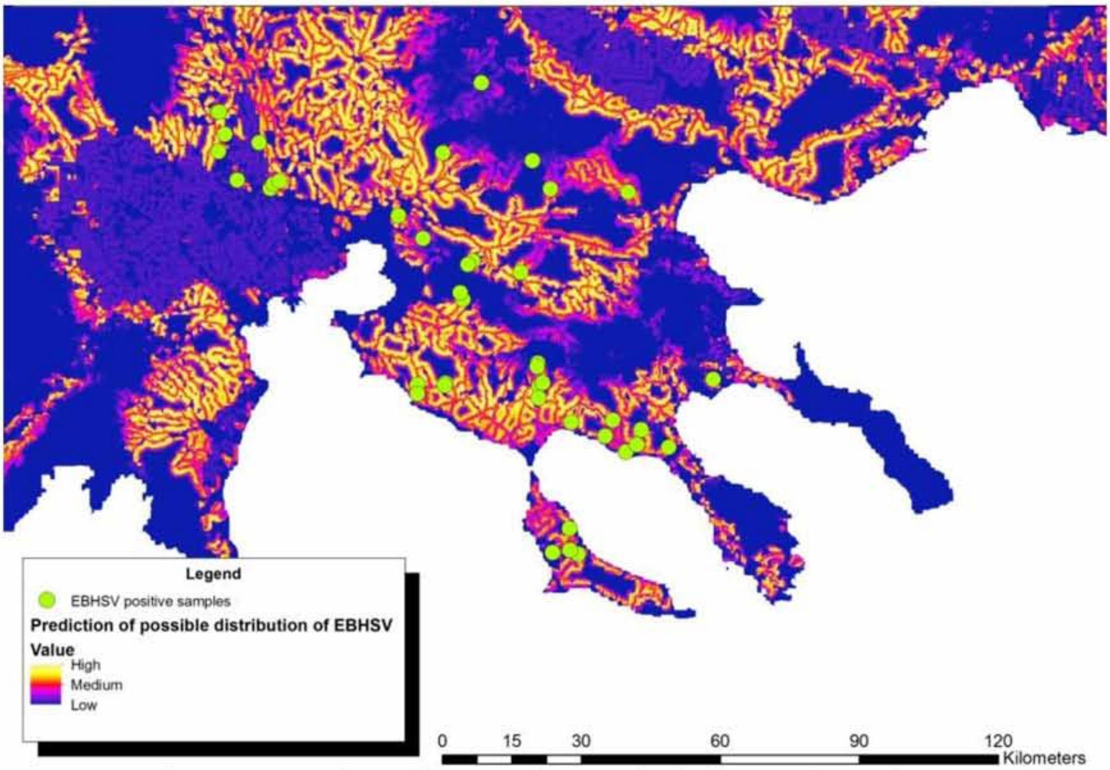

Fig. 3. *Prediction map of EBHSV prevelance. The bullets indicate the positive hares to EBHSV.*

3.4 Discussion

In our study, RT-PCR revealed 17.6% prevalence of EBHSV infection in hares older than 50 days old. The infection prevalence was found to be 17.9% during 2010-2011 and 17% during 2011-2012. This prevalence stability between the two hunting periods, indicates that the disease is in an endemic stability. The same holds true for the prevalence of infection between the dry-warm period (15/9-31/10) and the wet-cold period (1/11-10/1), thus permitting the pooling of the data. Moreover, the fact that EBHSV was present in the entire study area suggests that the disease system is in equilibrium, making thus possible the spatial analysis (Ostfeld et al. 2005).

These results are in great agreement with a previous study in which 85 hunting harvested hares were tested for EBHSV by RT-PCR and the infection prevalence was found to be 17.7% (Frölich et al. 2007). Regarding the seroprevalence, it is expected to be higher than the infection prevalence in areas where the disease is endemic. Indeed, in Northern Italy, 47.6% of the hares examined were found positive for EBHSV while 95% of them were found positive for antibodies against EBHSV (Scicluna et al. 1994). In other studies, the reported seroprevalence ranged from 36-

95%. Importantly, in Slovakia (Frölich et al. 2007) and France (Guitton et al. 2006), the reported seroprevalence differed significantly among the areas of the same country.

Hare population density is relatively low in many areas of Greece compared to other European countries (Sfougaris et al. 1999). According to Lavazza et al. (1999) the low hare density leads to a proportional reduction of EBHSV prevalence because of a reduction in the EBHSV transmission in these areas. The results of this study showed that the low hare densities cause a proportional reduction of EBHSV prevalence. In Germany, it has been reported that the bigger the hare harvest, the higher the percentage of infected hare (Riedel et al. 1991). Furthermore, in Britain, it has been reported that high mortality incidents due to EBHS, did not have an impact in the hare harvest (Duff et al. 1997). Syrjälä et al. (2005) found more mountain hares (*Lepus timidus*) dead by EBHS in areas with higher population densities. Similarly, Calvete et al. (2002) found a significant increase in the prevalence of RHDV antibodies in parallel with an increase in rabbit population density.

In low density hare populations less pathogenic or apathogenic EBHSV strains may be favored (Fouchet et al. 2009). In our study, EBHSV was present in areas with low population density. In such a case, the development of protective antibodies against EBHSV among young hares is expected to be low. The fact that only a few hares were found dead, can be attributed to the existence of less pathogenic or apathogenic EBHSV strains as it has been reported previously (Guitton et al. 2006, Frölich et al. 2007). Moreover, the existence of a chronic form of EBHS that persists in hares has been described (Frölich and Lavazza 2008, Billinis et al. 2005). In the chronic form of the disease, the virus may be complexed by antibodies and it is no more excreted, as it has been described previously for RHD (Barbieri et al., 1996). On the other hand, in Ormylia, the area with the higher hare population density among the areas studied, we found the higher prevalence. It is worth mentioning that in this area the introduction of allopatric EBHSV strains has been documented. The higher hare population density of the area may have contributed to the establishment of these allopatric strains, which probably have higher virulence to hares (Spyrou et al. 2013).

In hunting areas of Italy more dead hares by EBHS and a higher percentage of seronegative animals were detected in comparison with refuges (no hunting areas) where hare densities are significantly higher (Cammi et al. 2003, Paci et al. 2011). The seronegative animals are increased with the distance from refuges (Paci et al. 2011). In this study few hares were collected from refuges so any comparison with hunting areas was not possible. Moreover, any relation of prevalence with distance from refuges was not found. Hare counts in our study area showed that the hare densities are higher in refuges, this difference is about 20-50%, whereas in the case of Paci et al. (2011) the differences were higher. Additionally, in refuges of study area densities remain below the threshold of 8 hares/km², consequently hares dispersion from refuges to hunting areas may is not so extending, dispersers have lower survival (Avril et al. 2012) and also the practice of capture of hares and their translocation to hunting areas is not applied in Hellas, whereas in Italy is a common practice (Paci et al. 2011).

The high abundance of stray and shepherd dogs, stray cats, foxes (*Vulpes vulpes*), stone marten (*Martes foina*) and other predators in the study area (Birtsas et al. 2012) together with their predation to hares are considered to be an important limiting factor for hare population (Vlachos et al. 2006). This seems to be the main reason why hare populations located in suitable habitats and refuges present low densities. Consequently, in the majority of the areas, predation may constitute the main factor that keeps the hare density below the threshold of 8 hares/km². Therefore, the low hare density leads not only to the reduction of the pathogen transmission but also to the lack of development of protective immunity in the majority of the juveniles.

The presence of EBHSV in areas isolated from the mainland of Greece such as the island of Thasos and the Kassandra Peninsula, is indicative of the ease of the virus transmission. This finding is in agreement with a previous report in islands of Sweden and Denmark which raised the question about the role of birds in the transmission cycle of the virus (Syrjälä et al. 2005). The transportations and the vehicles have been also incriminated for the virus spreading (Chasey 1994). The infection prevalence was found to be increased near the paved road network. It is well known that the roads may contribute to the transmission of human pathogens in forested areas (Eisenberg et al. 2006) and plant pathogens (Jules et al. 2002). However, only a few studies have been conducted in mammals regarding the impact of roads in the prevalence of the infection in wild animals and livestock (Cooper et al. 2010). In a previous study, the introduction of RHDV in UK was attributed to the passive virus transmission through human traffic (Chasey 1994).

Hare killing by collisions with vehicles is a common fact in paved roads (Roedenbeck and Voser 2008) that has been also reported in Northern Greece (Platis and Sfougaris 2006). Importantly, EBHSV may remain infectious for 3–4 months in the field (Frölich and Lavazza 2008). Insects, corvids and other scavengers contribute to the spread of EBHSV around the roads by feeding on infected hares killed by vehicles (Asgari et al. 1998, McColl et al. 2002, Frölich and Lavazza 2008). The vehicle movement may also accidentally transfer flies, other insects and dust making thus possible the EBHSV transfer. Moreover, the dry Mediterranean climate conditions along with the fast vehicle moving may lead to the creation of aerosols which may carry the virus (Wobeser 2006).

In this study, significance of altitude is marginal with decrease of prevalence in higher altitudes. Frölich et al. (2001) refer that the mountainous areas of Alps and Jura may explain why in Switzerland EBHS had a delayed presence and was not reported to cause losses in hares until 2000. Similarly in Pyrenean Mountains Gortazar et al. (2007) did not report EBHS as an important disease for hare species. Possible explanations are that: 1) in mountainous areas the dispersion of hares is shorter due to the landscape resistance and other habitat characteristics, 2) in lower altitudes there are more mosquitoes and flies which contribute to virus transmission, and 3) in lowlands the distribution of hares is more clumped in patches that provide cover and food and thus the transmission between animals is easier.

3.5 Conclusions and management implications

To our knowledge**,** this is the first study about the ecology of Lagoviruses using a combination of GIS, Binary Logistic Regression and Mahalanobis distance. During the last years, the disease seems to have a low impact on the hare populations studied and it is in an endemic stability permitting its distribution study. EBHSV was found in all the areas studied although with different prevalence across landscapes. EBHSV prevalence is higher in areas with higher hare densities, nearer to paved roads and in lowlands. Habitat type, vegetation, temperature, precipitation, slope, aspect, human population, dog training areas, distance from livestock units and refuges did not have a significant influence to EBHSV prevalence.

Understanding the spatial distribution of pathogens is important for decision makers who need to apply measures for wildlife conservation and especially for hunted species with serious socio-economic importance (Joseph et al. 2013). Hare species in many areas have high conservation importance due to the increased genetic variability and geographical substructuring of populations (Mamuris et al. 2001, Antoniou et al. 2013). Hares have fundamental roles in natural processes as prey of threatened predator species (Delibes-Mateos et al. 2009) and are important sedentary hunted species (Papaspyropoulos et al. 2014).

In many areas hare has low densities and the increase of population is not a feasible task (Sfougaris et al. 1999, Smith et al. 2004, Delibes-Mateos et al. 2009). Consequently high densities >15hares/km seems to be impossible to be reached and the protection of population from EBHS through the development of protective immunity in young, is not a feasible option. This is a fact in our studied areas where several factors like the high pressure of predation and other mortality factors, including EBHS, do not permit the increase of hare population.

In these cases, managers should avoid measures that increase population in small areas within the broader landscape of low hare densities due to the fact that these areas will have high EBHSV prevalence and thus will act as dangerous zones of higher prevalence of pathogen. Moreover, this will probably influence the evolution of EBHSV with the conservation of more pathogenic strains which require higher host densities due to the higher mortality that cause (Pianka 2000, Fouchet et al. 2009). Thus, these areas can be characterized as EBHSV high prevelance Units (EBHSVU). Such Units may be farms with reared hares in cages or outdoor enclosures. Similarly, areas with intensive predator control and hunting prohibition for many years where high hare densities are developed may also act as EBHSVU.

In EBHSVU, hares may develop protective immunity, however they act as carriers and excretors and their natural dispersion for many km (Avril et al. 2011, Bray et al. 2007) or their translocation by humans, may probably lead to the disperse of the virus with consequences for the neighboring low density populations. This seems to happen in Italy where low seropositivity and high mortality caused by EBHS was associated to low hare densities in hunting areas whereas in the refuges where the seroprevalence and densities were higher, the mortality was lower (Cammi et al. 2003, Paci et al. 2011). Therefore, hare populations can be better protected by EBHS if their density distribution is as much uniform as possible, instead of clumped as it happens through intensive hunting or refuges for example. Consequently, a better hunting management including fewer refuges and more appropriate harvest rules will also provide protection from EBHS.

In a next step, it should be seriously considered that if these EBHSVU are near to paved roads and in lowlands, the prevalence and the virus transmission may be higher. Paved roads are usually selected as the boundaries of refuges in Greece. Moreover, high hare mortality has been reported in paved roads due to collisions with cars leading to carcasses which may act as source for virus spreading. The insects are active in the Mediterranean lowlands nearly all year round and their role in the virus transmission may be more important than in continental Europe (Asgari et al. 1998, McColl et al. 2002). Therefore, managers should not prefer or should avoid refuges and hare farms to be near the paved roads and in lowlands. This is an important point which should be taken into account in the design of refuges and protected areas as well as in the environmental assessment of paved roads

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The prediction modelling of possible occurrence of pathogen seems to be an effective tool that informs the managers about the risk areas of disease. Thus, preventive measures can be taken for the mitigation of impacts to population such as the restriction of translocations from and to risk areas and the population increase in areas of low risk. Moreover the data obtained can contribute in future sampling design. For example samples collected from areas near paved roads are expected to have higher prevalence. A future research on EBHSV strains and their relation with virulence, hare population density and other environmental parameters will be of great interest and will provideanswers on evolutionary ecology.

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4. DEMOGRAPHY, PHYSIOLOGY AND EBHS VIRUS PREVALENCE: IS THERE AN ASSOCIATION FOR THE BROWN HARE?

Abstract

Epidemiological studies of disease ecology typically ignore the influence of host sex, age, nutritional condition and immunocompetence factors. However, this can lead to shortcomings and incorrect conclusions regarding the mechanisms of pathogen transmission and prevalence in wild animals. In many European countries, European Brown Hare Syndrome virus (EBHSV) causes a disease lethal to brown hares (*Lepus europaeus*). Associations of EBHSV infection, sex, age, body condition and spleen mass were studied in hares collected during the hunting season. Data showed a sex-biased prevalence with twice as many males infected with EBHSV than females, indicating a sexual dimorphism in disease exposure or susceptibility. EBHSV infection was not related to hare body condition and a higher body condition was found in males than in females. Adults had a higher ratio of infected animals than young but this difference was not found to be statistically significant. Adults also had higher spleen mass thus indicating past infections. An enlarged spleen was more frequently found in hares positive for EBHSV and a negative relation was found between spleen mass and body condition thus indicating energy consumption for immune defense investment. This study into the ecology of EBHSV is one of the few that examine the relevance of individual risk factors in understanding patterns of viral infections in natural populations of wild animals.

4.1 Introduction

Understanding the ecology of the immune system is important for studies on the prevalence, transmission, dynamics and distribution of infectious diseases; issues which should be examined in combination with the different susceptibility or exposure of individuals in their natural environment (Beldomenico & Begon, 2010). Differences in sex, age, body condition and immunity between individuals may influence proneness to infection (Klein, 2000; Wobeser, 2007).

Males are typically more mobile and have more frequent contact with other individuals compared to females. In addition, differences in stress level during the reproduction period may influence differentially immunosuppression and susceptibility (Folstad & Karter, 1992; Klein, 2000; Cross *et al.*, 2009; Scantlebury *et al.*, 2010; Bordes *et al.*, 2012). A case was recorded for chamois (*Rupicapra rupicapra*) where the prevalence of brucellosis was higher in males because they grazed livestock-contaminated low pastures more frequently than females (Ferroglio, 1998). In wood mouse (*Apodemus sylvaticus*), females seem to invest more in immune defense, but also appear to be potentially more tolerant of helminth diversity. Prevalence in adults may be higher due to longer exposure time, but may be lower due their faster recovery as adults may already be immune or have higher mortality (Cross *et al.*, 2009).

Animals with good body condition may have lower prevalence than undernourished or stressed individuals. Usually, lower fitness is referred as a consequence rather than a cause of infection (Beldomenico & Begon, 2010). Pregnant and lactating females of little red flying foxes (*Pteropus scapulatus*) that showed evidence of nutritional stress had higher seroprevalence to the Hendra virus (HeV), suggesting that habitat degradation that alters flying fox food sources and may increase HeV infection and transmission (Plowright *et al.*, 2008). Similarly, Lello *et al.*, (2005) found that mass of abdominal fat and total body mass of the rabbit (*Oryctolagus cuniculus*) were negatively related with myxoma virus infection. In case of parasites, Iason and Boag (1988) found that the intensity of infection from the intestinal helminth *Trichostrongylus retortaeformis* had no influence on weight and fatness of mountain hares (*Lepus timidus*), although Alzaga *et al.*, (2008) found that the burden of *Taenia pisiformis cisticercus* influences negatively the body condition of Iberian hares (*Lepus granatensis*).

It is known that immunological defenses are costly to the host and individuals in good physical condition should be able to invest more in lymphocyte production and storage (Lochmiller & Deerenberg, 2000; Schulte-Hostedde & Elsasser, 2011). Lymphocytes are used to fight infection and the spleen is the main organ involved in their production. Vicente *et al.*, (2007) found that spleen mass of red deer (*Cervus elaphus*) is positively related to body condition and negatively to nematode infections. On the contrary, in the American mink (*Neovison vison*) the richness of parasites that infect the animal causes an increase of spleen mass (Schulte-Hostedde & Elsasser, 2011). In house mouse (*Mus musculus*) strains, immunocompetence was assessed indirectly by spleen mass, and directly by the phytohaemagglutinin (PHA) test. The results showed no relationship between the resistance/susceptibility to nematode burdens and the level of PHA response or the size of the spleen between strains (de Bellocq *et al.*, 2007). In cases of viruses, male grey squirrels (*Sciurus carolinensis*) with larger spleens were more likely to be seropositive to squirrelpox virus (McGowan *et al.*, 2014).

Larger spleens are observed in rabbits and hares that have died from Lagoviruses (Billinis *et al.*, 2005; Frölich & Lavazza, 2008; Abrantes *et al.*, 2012). This group includes the Rabbit Hemorrhagic Disease Virus (RHDV) and the European Brown Hare Syndrome Virus (EBHSV). Cabezas *et al.*, (2006) found that rabbits with better body conditions at the time of vaccination had greater immune response against RHDV and myxomatosis. However, more information on the prevalence of these Lagoviruses and the demographical and physiological factors of their host is limited (Frölich & Lavazza, 2008; Abrantes *et al.*, 2012).

EBHS is a significant disease for hares (*Lepus* spp. Wibbelt & Frölich, 2005), causing high mortality rates in infected animals, whereas morbidity can reach 100% (Zanni *et al.*, 1993 & 1995; Duff *et al.*, 1994; Drews *et al.*, 2011). The disease has not been observed in leverets younger than 40-50 days, however juveniles younger than 2-3 months may contract the infection but do not develop the disease (Zanni *et al.*, 1993; Scicluna *et al.*, 1994; Lavazza *et al.*, 1999; Drews *et al.*, 2011). In the case of EBHS, and according to the above, it could be predicted that: a) adults and males have higher probabilities of infection, b) spleen mass (controlled for age) is heavier in infected hares, c) spleen mass is related to body condition with a positive or negative relation that reflects the cost of maintaining an effective immune system, and d) an interaction exists between EBHSV infection and body condition with infected hares having lower body conditions, however infected hares that are collected alive are those with better body conditions and can overcome disease. The aim of this study was to investigate possible associations between sex, age, body condition, and spleen mass in EBHSV infection (Fig. 1).

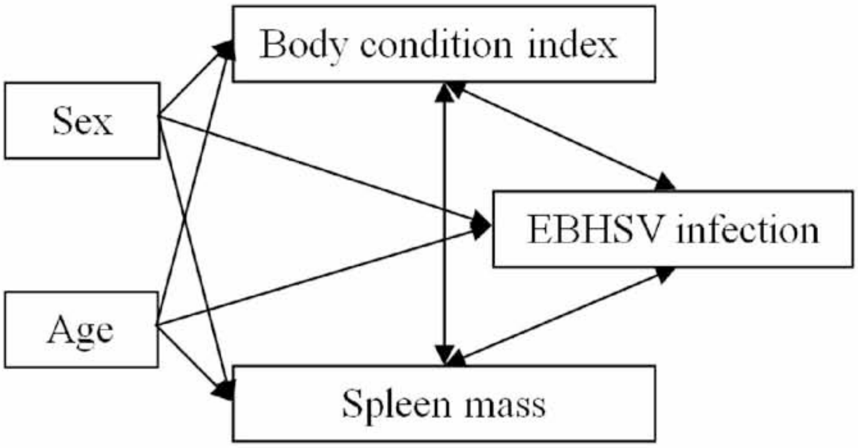

Fig. 1. *Hypothesized path of the influences examined in this study. Arrows with two heads denote possible interactions according to the literature.*

4.2 Materials and Methods

The study was conducted in the Prefectures of Chalkidiki and Thessaloniki in an area of 6,601 km². The area is classified as having a Mediterranean-type climate with dry, hot summers and mild winters. The highest temperature ranges between 23-34°C during summer and the lowest between 4-19°C in winter.

Hares were harvested by legally licensed volunteer hunters and several animals were found dead within the study area. The authors declare that this study is based on legal hunting methods according the Hellenic Hunting Law (200148/2833/06-08-2010, Decision of the Ministry of Agriculture). A few hours following collection, the animals were examined for sex determination then dissected. Females were classified according to their three reproductive states: a) lactating females with milk in mammary glands, b) pregnant females characterized by the presence of embryos, and c) not pregnant.

One eye of each animal was preserved in 10% formalin, while the other organs were frozen five to eight hours following death. To determine the age of each individual hare, the crystalline lens was weighed, however in absence of eye, Stroh's method on the ossification stage of epiphysis in the ulna was used (Suchentrunk *et al.*, 1991). Thus, the hares were divided into two age classes: a) young (hares younger than 6-7 months old with dried eye lens weights <235mgr), and b) adults (older than 7-8 months old with dried eye lens weights >235mgr) (Suchentrunk *et al.*, 1991).

Hare body condition was determined using the kidney fat score on a scale 0-5 (Bonino & Bustos 1998; Banks *et al.*, 1999; Jennings *et al.*, 2006). As an index of immunity, fat around the spleen was removed carefully and the spleen mass (±0.01 g) was measured using an electronic digital scale. The parasitic burden of a subsample was examined and was found to be at low levels in the majority of the animals (Diakou *et al.*, 2014).

Tissue samples from liver were obtained and stored at -80°C until analysis. Total RNA was extracted from all liver samples using the PureLink™ RNA Mini kit (Invitrogen Corporation, Carlsbad California, USA) according to the manufacturer's instructions. RT was performed using reagents supplied with the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen Corporation, Carlsbad California, USA). A preliminary mixture containing 2 μl of RNA extraction product, 1 μl of dNTP mix (10 mM), 5 μl of Random hexamers (50 ng/μl), and 2 μl of DEPC-treated water was incubated for 5 minutes at 65°C, and was then placed on ice for at least 1 minute. Subsequently, 9 μl of a 2X reaction mix containing 2 μl of 10X RT buffer, 4 μl $MgCl_2$ (25 mM), 2 μl DTT (0.1 M) and 1 μl RNaseOUT™ (40 U/μl), were added to each RNA/primer mixture. After incubation at room temperature (~25°C) for 2 min, 1 μl SuperScript™ II RT (50 U/μl) was added to each tube followed by incubation at room temperature (~25°C) for 10 min. Reverse transcription reactions were carried out at 42°C for 50 minutes and they were terminated by heating at 70°C for 15 min. After chilling on ice, 1 μl RNase H (2 U/μl) was added into each reaction mixture and was then incubated at 37°C for 20 min. The reactions were stored at -20°C pending PCR.

PCR amplification was carried out in a final volume of 20 μl reaction mixture containing 2 μl of the cDNA reaction, 2 μl of 10X PCR Buffer, 2 μl dNTP mix (2,5 mM), 0,8 μl $MgCl_2$ (50 mM), 0.2 μl Taq DNA Polymerase (5 U/μl), 20 pmoles of each primer and up to 20 μl DEPC-treated water. Primers HEF 5'-CCGTCCAGCATTCGTCCTGTCAC-3' (nt1 795–1817) and HEB 5'-CATCACCAGTCCTCCGCACCAC-3' were selected from the VP60 gene of EBHSV (nucleotides 6423 to 6687 on the complete genome sequence of EBHSV GD, accession no. Z69620). The cycling conditions were initial denaturation at 94°C for 9 min followed by 35 cycles consisting of denaturation at 94°C for 45 sec, annealing at 60°C for 45 sec and extension at 72°C for 1 min, followed by a final extension at

72°C for 7 min. Amplification products were subjected to electrophoresis in 2% agarose gel stained with ethidium bromide (0.5 μg/ml) and visualized under ultraviolet light. Product sizes were determined with reference to a 100-bp molecular-weight ladder. The expected RT-PCR product size was 265bp.

Statistical analyses

All statistical tests were performed with IBM SPSS 21.0 for Windows (SPSS Inc., Chicago, IL, USA). Due to the missing values of examined variables for some samples, initially the basic statistics (Chi-square test, independent samples t-test) were used with more samples, and then hares for which all variables were measured were included in univariate General Linear Models (GLM). Levene's test was used to check the homogeneity of variances (Zar, 1996). GLM was used to explore the effect of age, sex, and EBHSV on body condition. A second data set including sex, age and EBHSV as fixed factors, and body condition as a covariate, was used to explore the effect on spleen mass. All data are expressed as arithmetic mean ±SE and all tests are two-tailed. Results are considered significant at $P<0.05$ and tending towards significance at $P<0.1$.

4.3 Results

General findings

Leverets younger than 50 days old were negative to EBHSV were excluded from the analysis and being in an early growth phase and thus not susceptible to disease. Moreover, one hare that had been found dead in the field was excluded from the study as it exhibited indications of poisoning with an extremely high spleen mass of 34 gr and viral and parasitological examinations did not show any infection by pathogens. In total, 259 samples were included in this study. All samples had been collected during the hunting periods (15/9-10/1) of 2010-11 and 2011-12.

The percentage of hares positive to EBHSV was 17.93% in the 2010-11 and 17% in the 2011-12 seasons. This difference was not found to be significant (χ^2=0.043, P=0.828), and similarly no differences were found for body condition and spleen mass (P>0.2). Moreover, there was no difference between the dry-warm period (15/9-31/10) and the wet-cold period (1/11-10/1), percentage of hares positive to

EBHSV (χ^2=0.003, P=0.952), or body condition and spleen mass (P>0.2). Consequently, no significant differences were found in all the variables examined between years and periods, therefore permitting the pooling of the data and thus improving the study of effects due to demographic and physiological factors.

EBHSV prevalence analyses

Juveniles younger than 6-7 months old constituted a percentage of 66%. In adults the ratio of positive animals was higher (19.7% for adults vs. 15.6% for young) but this was not significant (χ^2=1.37, P=0.549). The sex ratio was female/male 1:34, and this did ratio not differ between young and adults (χ^2=0.093, P=0.76). A higher prevalence of EBHSV was found in males with 25.81% positive to the disease compared to 13.6% of females approaching significance (χ^2=3.51, P=0.061). Among the females, 15.2% was pregnant or lactating, although the reproductive state had no influence on EBHSV prevalence or body condition (P>0.1).

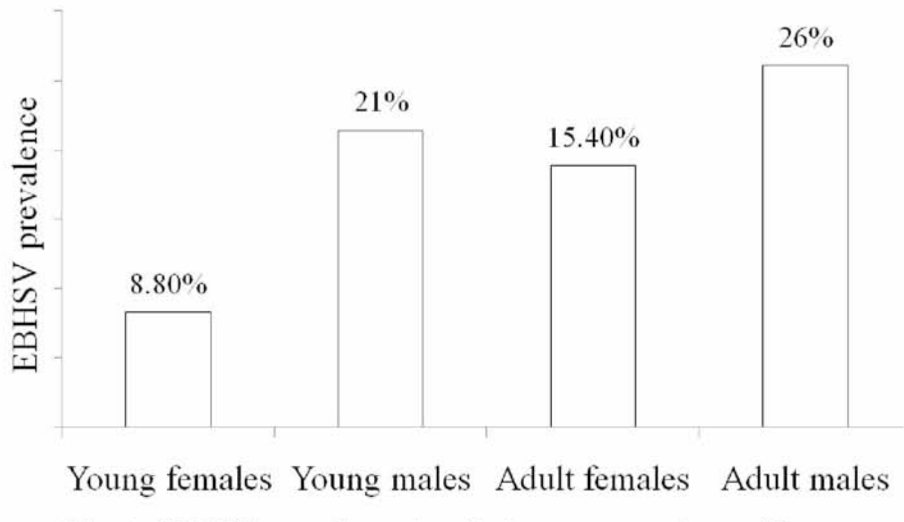

Fig. 2. *EBHSV prevalence in relation to age and sex of hares.*

Body condition analyses

No significant differences in body condition were observed among hares positive or negative to EBHSV (t=0.19, P=0.853, df=186), although there was a greater variability in body condition in positive hares (Fig. 3a). Between juveniles and adults the difference in body condition was not significant (t=0.19, P=0.849, df=140), whereas between the two sexes, males had a higher body condition. In males, the mean body condition was 1.57±0.16 SE and 1.24±0.12SE in females, and this difference approaches significance (t=-1.74, P=0.084, df=163).

The GLM revealed that the spleen mass is a significant covariance (P=0.025-0.051, Table 1), and the difference in body condition between males and females approaches significance (P=0.053-0.085). EBHSV infection and age have no significant effect (P>0.2).

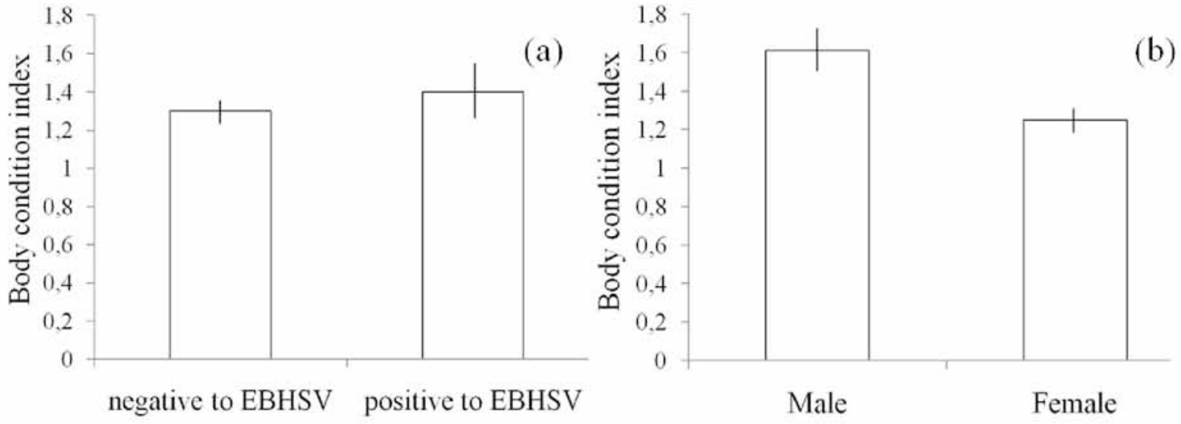

Fig. 3. *Mean body condition index and standard error in relation to: (a) EBHSV infection and (b) sex of hares.*

Table 1. Results of two GLMs on effects of EBHSV, sex, age and their interactions on body condition of hares.

Source of variation	Model[a]	df	F	P
Spleen	1	92	3.934	**0.051**
EBHSV	1	92	1.659	0.201
Sex	1	92	3.906	**0.053**
Age	1	92	0.238	0.627
EBHSV*Sex	1	92	0.901	0.345
EBHSV*Age	1	92	0.639	0.426
Sex*Age	1	92	1.107	0.296
EBHSV*Sex*Age	1	92	2.208	0.141
Spleen	2	114	5.195	**0.025**
Sex	2	114	3.014	**0.085**

[a]Model 1 examined the effect of EBHSV infection, sex, age and spleen mass as covariance on body condition. Model 2 examined the effect of sex and spleen mass on body condition.

Spleen mass analyses

Age was significant for spleen mass as in adults the mean mass was 3.65±0.5SE whereas in juveniles it was 2.16±0.23SE (t=3, P=0.003, df=104). Pregnant or lactating females showed higher spleen masses in comparison with the non-pregnant females, however, the sample was not sufficiently large. Sex did not influence spleen mass (t=-0.38, P=0.706, df=124).

Hares with a heavier spleen have a slightly lower body condition (Pearson's r = -0.187, P=0.04). Hares positive to EBHSV had spleen masses of 3.81±0.75SE and hares negative to EBHSV had masses of 2.43±0.19SE. This difference was found to be statistically significant (t=2.6, P=0.01, df=134).

Taking the most important independent variables into account, GLM revealed body condition is a nearly significant covariance of spleen mass (P=0.078, Table 2). The difference in spleen mass between hares positive and negative to EBHSV is nearly significant (P=0.058), whereas the influence of age is significant (P=0.014).

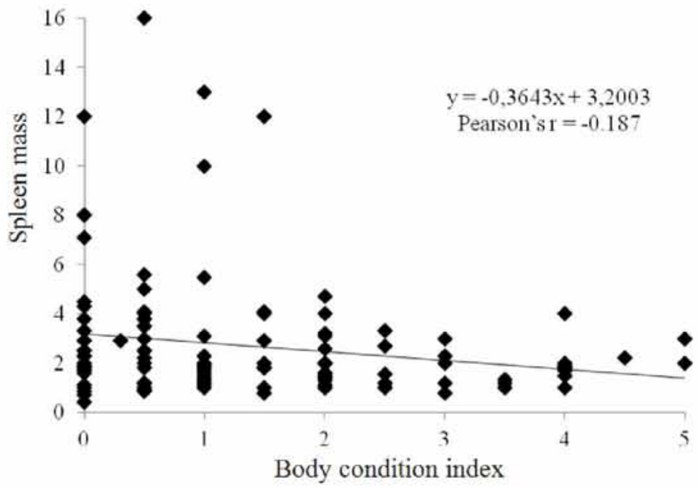

Fig. 3. *Spleen mass (gr) in relation to body condition index.*

Table 2. Results of GLM on effects of EBHSV, age and their interactions on spleen mass of hares.

Source of variation	df	F	P
Body condition	98	3.17	**0.078**
EBHSV	98	3.692	**0.058**
Age	98	6.28	**0.014**
EBHSV* age	98	0.43	0.514

4.4 Discussion

The prevalence of EBHSV was found to be the same in both study years. This prevalence stability indicates that the disease is in an endemic stability phase. The same holds true for body condition and spleen mass. No difference was found in the above three variables between the dry-warm period (15/9-31/10) and the wet-cold period (1/11-10/1), thus permitting pooling of the data.

EBHSV prevalence showed an increase with age, but this increase was not found significant. Guitton *et al.*, (2006) refer a higher seropositivity to EBHS in adults than in young. In this study the PCR method was used to examine virus presence, contrary to the antibody detection method used by Guitton *et al.*. The PCR method probably mitigates the influence of age because some adults may have already been immune thus preventing a new virus infection (Cross *et al.*, 2009), consequently with PCR these hares appeared as negative whereas when using ELISA they are seropositive. Healthy hares may have antibodies, but in the absence of virus particles (Cammi *et al.*, 2003; Drews *et al.*, 2011). In chronic forms of the disease, the virus may form complexes with antibodies and is no longer, as described in cases of RHD (Barbieri *et al.*, 1996). For example, in Northern Italy, 47.6% of samples were found to be positive to the virus, whereas 95% of samples tested positive for EBHSV antibodies (Scicluna *et al.*, 1994).

The influence of sex was more important than age because twice as many males were found to be infected with EBHSV than females, indicating a sexual dimorphism in disease exposure or susceptibility. A similar pattern was found also in Italy where more young males were seropositive to EBHS than young females (Paci *et al.*, 2011). In France, more males than females died of EBHS, and if the sex ratio towards females is taken into account, this finding appears to be significant (Lamarque *et al.*, 1996). Immunosuppression, stress and other sex-related factors may explain this difference, because it is common for male mammals to be more susceptible to disease (Folstad & Karter, 1992, Scantlebury *et al.*, 2010, Bordes *et al.*, 2012). The 'immunocompetence handicap hypothesis' refers that androgens may produce immunosuppressive effects reducing resistance to parasites (Folstad & Karter, 1992). In this research, this hypothesis cannot be verified as sampling did not take place in the main reproductive season of hare and differences were also found for young animals that had not yet reproduced.

One more probable explanation seems to be that males are more mobile than females. Bray *et al.*, (2007) and Avril *et al.*, (2011) showed that natal dispersal was sex-biased, with a greater propensity to disperse in young males. Moreover, males are more active and have larger home ranges than females (Zaccaroni *et al.*, 2013) and chase each other to attain dominance (Hansen, 1996; Flux, 2009). Hansen (1996) found that female hares spent more time feeding and resting whereas males spent more time moving around, exploring, coupling and demonstrating agonistic behavior. These facts increase the contact of males with the external environment and other hares, and thus increase the probability of infection. According to Frölich & Lavazza (2008), "EBHSV is highly robust, resisting acid of pH3, and may remain infectious for 3-4 months in the field".

The dispersal of young takes place mainly when the hares reach the age of four to six months (Bray *et al.*, 2007; Avril *et al.*, 2011) and when they have lost their ability to become immune to a great extent (Zanni *et al.*, 1993; Scicluna *et al.*, 1994; Lavazza *et al.*, 1999; Drews *et al.*, 2011), thus mortality may be higher in males. It is characteristic that the female/male ratio of 1.34 is similar to that of 1.29 found in a previous study in the same area (Retzepis *et al.*, 2006), but is slightly higher than that of other studies (Flux 1967, Wasilewski 1991; Bonino & Montenegro 1997), and even higher than ratios found in the 1960s (Pielowski 1969), before the influence of EBHS. One explanation is that different susceptibility and mortality to diseases between the two sexes may contribute to a differentiation of the sex ratio between areas and years.

Body condition was also found to be significantly higher for males than females, in accordance with a previous study in the area; however the difference was not statistically significant (Retzepis *et al.*, 2006). In general, females are fatter than males (Bonino & Bustos, 1998), although in this research hares were collected after the breeding season when females have lost their fat reserves, and at the beginning of the next breeding period when males begin to accumulate energy before females (Pepin, 1987; Bonino & Bustos, 1998).

Infection by EBHSV did not significantly influence body condition, and susceptibility to EBHSV was not influenced by body condition. Brown hares found dead by EBHS in Finland were mostly of normal or moderate body condition (Syrjälä *et al.*, 2005). Similarly, in Italy, hares with acute hepatosis by EBHS were in good nutritional condition, as indicated by fat deposits, and their stomachs were well filled with food (Poli *et al.*, 1991). In its acute form, EBHS has a short clinical course, and affected animals die mainly within 1 to 2 days. Consequently, the virus allows no time for serious impact on body condition (Poli *et al.*, 1991). However, in these studies an index of body condition or weight was not applied. According to Gavier-Widen and

Morner (1993), the body weight of hares with EBHS pathologic findings appeared to be lower than that of healthy hares. Obviously, this weight loss is more frequent when the disease is in its subacute and chronic form (Gavier-Widen, 1994; Billinis *et al.*, 2005). In this research, only one weakened young hare was caught by dogs in January. This animal was infected with EBHSV and its body condition index was zero. Two more animals found dead and infected by EBHS during the study, had good muscle condition however, the body condition index was low at 0 and 0.5. Consequently, cases of subacute or chronic forms of the disease that seriously impact body condition appear to be limited in this study area.

EBHSV infection usually causes the acute form of the disease or some animals are strong with increased body conditions and this assists their recovery. Thus, these animals may be infected but with a higher body condition than non-infected hares. Moreover, the virus may be apathogenic, something that is dependent also on the EBHSV strains present in the area (Guitton *et al.*, 2006). In the study area, alien strains of EBHSV have been introduced by released hares, possibly resulting in different impacts than those caused by the indigenous virus strains (Spyrou *et al.*, 2013). This differential influence caused by the different responses to the virus and the different EBHSV strains may also cause the characteristic higher variability in body condition seen in positive hares compared to the negative animals (Fig. 3a).

In contrast with body condition and fat accumulation, disease infection causes a more lasting and direct effect on the immune defense investment of animals as shown by the spleen mass increase (McGowan *et al.*, 2014) of hares infected with EBHSV (Syrjälä *et al.*, 2005). Enlarged spleens could be the result of a present or past infection that promoted the increased production and storage of lymphocytes (John, 1994; Bordes *et al.*, 2012; McGowan *et al.*, 2014), and thus are less dependent on the individual's body condition (John, 1994). Adults and hares infected with EBHSV had higher spleen masses and hares with a higher spleen masses had lower body condition. Scantlebury *et al.*, (2010), found that spleen mass was negatively correlated with body condition in grey squirrels. Immune function and body condition are associated (Demas & Sakaria, 2005; Bachman, 2003) and from this study it can be concluded that more energy is required to support the immune system in hares and thus less fat is deposited onto the kidneys indicating lower body condition.

4.5 Conclusions

A sex-biased prevalence was found with double the number of males infected with EBHSV than females, indicating a sexual dimorphism in disease exposure or susceptibility. Consequently, males may have higher mortality that appears to contribute to a sex ratio biased towards females. The few pregnant and lactating females collected did not show a significantly higher risk of infection. More adults were found infected than young, although this was not found to be significant. Parallel monitoring of seroprevelance would give more seropositive adults. However, adults had a higher spleen mass indicating past infections from EBHSV and other pathogens. Enlarged spleens were more frequently found in positive to EBHSV hares, and a negative relation was observed between spleen mass and body condition indicating energy consumption for immune defense investment; although in Mediterranean ecosystems hares do not face food shortages that could cause nutritional stress (Sokos *et al.*, 2015). EBHSV prevalence was not related with hare body condition, thus indicating a more complex relationship between pathogen influence and host response. In conclusion, the research identified that female hares appear to have lower infection risk than males, and hares with higher body condition appear to have higher probability to overcome the disease. The data provide support for the hypothesis that males favour investment in dispersion and mating at the expense of disease risk and immune function. This study highlights the value of disease studies in natural populations to understand drivers of pathogen infections that should be more widely acknowledged in disease ecology research.

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5. HARE ECOLOGY AFTER A HABITAT DISTURBANCE: THE CASE OF WILDFIRES

Abstract

Ecosystem disturbances, such as wildfires, are driving forces that determine conservation measures. Species respond differentially in wildfires, having diverse post-fire population evolution. We report on the responses of brown hare (*Lepus europaeus* Pallas, 1778) to wildfires in Mediterranean ecosystems. Hare relative abundance, age ratio, diet quality, body condition and diseases were determined. Fire influence on vegetation was calculated at a micro-scale level. Hare abundance was lower the first year after the wildfire in burned areas compared to unburned. This difference was more prominent in intensely burned area where the wildfire destroyed completely the woody vegetation. The reverse phenomenon was found in the second year when hare abundance was higher in burned areas. Higher hare abundance in burned areas continued in the third and fourth years. In the fifth and sixth years after wildfire no significant difference was found in abundance between the two areas. At a micro-scale level, higher numbers of hare faeces were counted in places with higher wildfire influence on vegetation. The analysis of the age ratio showed the existence of more juveniles and near adults in burned areas, with a stable presence of neonates, indicative of a lower mortality of juveniles in burned areas. This study demonstrates that severe wildfires decrease only temporally brown hare abundance, the species recovers rapidly and has higher abundance in burned than unburned areas for few years. Decrease of predation in burned areas was the most plausible explanation for our findings.

5.1 Introduction

The abandonment of traditional land uses in many Mediterranean areas has led to the growth of continuous forests and shrublands that are susceptible to wildfires (Moreira et al. 2011). Wildfires can increase landscape heterogeneity by fragmenting continuous blocks of dense wooden vegetation (Vazquez and Moreno 2001). However, in some cases, wildfires are so extensive and severe that they decrease the structural diversity and homogenize the landscape (Vazquez and Moreno 2001; Moreira et al. 2011).

Wildfires influences on wildlife species can be defined as either short-term or long-term (Quinn 1994). Short-term influences are displacement (Koprowski et al. 2006) and animal death directly caused by wildfire (Haim and Izhaki 1994) or indirectly caused through predation, starvation and reduced reproduction success by habitat degradation (Haim and Izhaki 1994). In the long-term, the evolution of an animal population after wildfire depends on parameters such as abundance of survivors, immigration, habitat, pathogens, predators and competitors recovery (Quinn 1994; Haim and Izhaki 1994; Torre and Díaz 2004).

According to Quinn (1994) there are three basic types of post-fire population responce in mammals: 1) species that are disappeared after wildfire and reoccupy the area only after plant community has returned to advanced stage of succession, 2) generalist species which have low abundance before the wildfire, and increase their abundance for a period after the wildfire, and 3) stenotopic species that appear for first time in the area in a later stage of succession after wildfire. All species of mammals do not belong entirely to one of the three types of post-fire population evolution, but most species are better classified to one type than another (Quinn 1994).

The effects of wildfires on some mammals are well studied (e.g. Torre and Díaz 2004), but studies on lagomorphs are limited. Keith and Surrendi (1971) found more *Lepus americanus* in the burned area one year after the wildfire. In savannah grassland in East Africa, Ogen-Odoi and Dilworth (1984) determined that hare species (*Lepus* spp.) and medium-sized predators increased by over 300% and 124%, respectively, three months after prescribed burning. However, frequent prescribed burning can influence negatively *Lepus flavigularis* in Mexico (Lorenzo et al. 2011).

Hare species (genus *Lepus*) have never been subject to an in-depth study, although, hare species: 1) in many areas have low population densities (Delibes-Mateos et al. 2009) with high conservation importance due to the increased genetic variability and geographical substructuring of populations in Mediterranean region (Antoniou et al. 2013), 2) have fundamental roles in natural processes as prey of threatened predator species (Delibes-Mateos et al. 2009), and 3) are important sedentary hunted species (Papaspyropoulos et al. 2014). Moreover, legislation is inconclusive about hunting management after wildfires in many Mediterranean countries. Thus, hare hunting after a wildfire is usually determined by local agencies without adequate scientific documentation, and the hunting is usually banned for a period of one to ten years after wildfires (Zamora et al. 2009, Papaspyropoulos et al. 2014). These hunting prohibitions have important economic and ecological impacts (Zamora et al. 2009; Papaspyropoulos et al. 2014).

In this study adjacent burned and unburned areas were compared on hares: (1) relative abundance, (2) age ratio, (3) diet quality, (4) body condition, and (5) pathogens. Moreover, we investigate if the severity of wildfire influences hares at a micro-scale level. The survey of population for six years following wildfire gives information about hare population evolution, whereas the measured demographic, dietary and disease parameters contribute to interpretation of hare abundance results.

5.2 Materials and Methods

Study sites

The study was conducted in burned and adjacent unburned areas in the Kassandra Peninsula of Chalkidiki Prefecture and in Lagada of Thessaloniki Prefecture (Fig. 1). The climate of both areas is classified as Mediterranean with hot and dry summers. In Kassandra the mean annual precipitation is 581 mm, the mean annual air temperature is 16.3 ℃ and has altitudes ranging from 150-280 m a.s.l.. In Lagada, the mean annual precipitation is 588 mm, the mean annual air temperature 12 ℃ and has altitudes of 200-520 m.

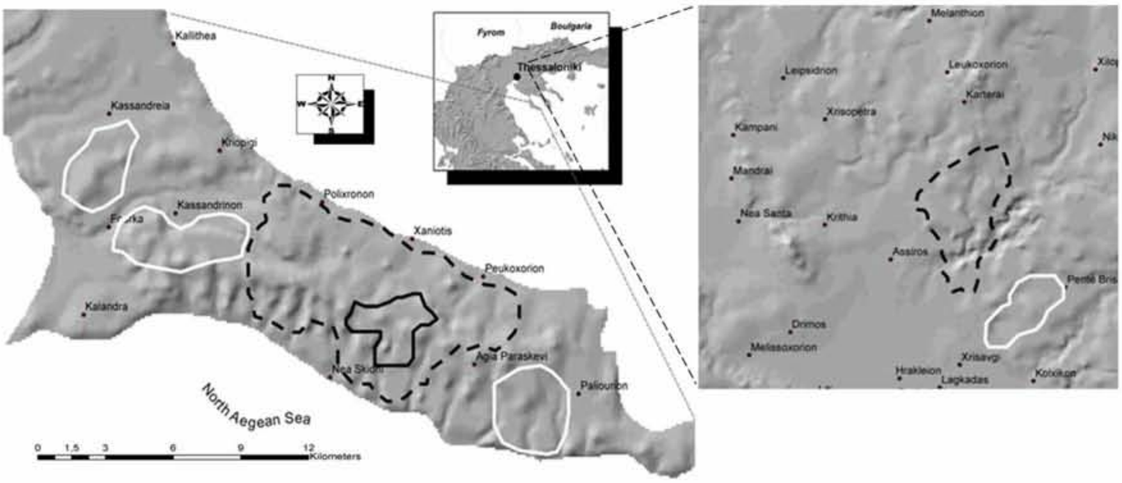

Fig. 1. The two study areas in Kassandra (left) and Lagada (right). The dashed black line indicates the moderately burned areas and the solid black line shows the intensely burned area. The solid white line shows the neighbouring unburned areas where hares where counted.

Kassandra Peninsula is covered mainly by forest vegetation of Aleppo pine (*Pinus halepensis* Mill) and broadleaved shrubs (*Quercus coccifera* L., *Smilax aspera* L., *Cistus* spp., *Pyrus spinosa* Forssk.), while there are also scattered agricultural fields, mainly comprising olive groves, cereal and a few vetch crops. Lagada area is covered mainly by broadleaved shrubs (*Quercus coccifera* L. and *Pyrus spinosa* Forssk.) and secondly by plantations of *Pinus brutia* Ten., while there are also scattered cereal fields. The woody plants of both areas regenerated quickly after wildfires and shrubs re- sprout reached the height of about half a meter in the first year after the wildfire

Wildfire occurred in Kassandra on 21/8/2006 and burnt an area of 68.7 km^2. In Lagada, the wildfire broke out on 27/7/2007 and burnt an area of 29.17 km^2. Due to very strong winds, wildfire severity was high with intensive crowning in both areas. In the centre of the burned region of Kassandra there is an intensely burned area of 18.3 km^2 where nearly all trees and shrubs were burned, whereas the remaining 50.4 km^2 is a moderately burned area with unburned stands (of about 0.1-0.5 ha), creating a mosaic of burned and unburned patches. Lagada is characterized as a moderately burned area. During the first year after the wildfire most of the burned trees were felled in both areas. Some trunks were removed while others, together with their branches, were used for soil-erosion barriers.

The study area of Kassandra stretches along the ridge of the peninsula and the unpaved forest road. The road has a total length of 22 km: 8.5 km passing through unburned areas, 9.5 km through moderately burned area, and 4 km passing through intensely burned area (**Fig. 1**). In Lagada the unpaved forest roads have a total length of 27 km: 12 km passing through unburned area and 15 km through moderately burned area.

Within both areas, hunters are interested mainly in hare and migratory bird quarries. The hunting of red fox (*Vulpes vulpes* L., 1758) and stone marten (*Martes foina* Erxleben, 1777) is permitted but they are rarely hunted (Papaspyropoulos et al. 2014). After the wildfire, hunting was prohibited throughout the Kassandra Peninsula until 25/11/2006 that is for about three months. After this date, hunting was permitted only in the unburned hunting area of the Peninsula. In the burned area, prohibition continued for two years after the wildfire and hunting was permitted again during the third year. In Lagada, hunting was prohibited in the burned area and 200 m around the burned area. Hunting dog training was permitted in the area 3.5 years following the wildfire, mainly to facilitate the adaptation of hares to hunting. Local hunters preferred to use this area only as dog training zone and thus hunting prohibition lasts until today.

Study design

We studied the influence of wildfire on hares at three levels: 1) vegetation and diet quality, 2) age ratio, body condition and diseases, and 3) hare abundance. The above parameters were estimated in burned and adjacent unburned areas of similar pre-fire vegetation and topography and pair-compared. The comparison of abundance between burned and unburned areas is a broad approach used in post-fire studies on the influences of wildfire on mammals (e.g., Borkowski 2004; Torre and Díaz 2004). Additionally, hare population trends are, at least locally, similar (Marboutin and Peroux 1996), so that differences in abundance can be attributed to the independent variables examined, which in this case is wildfire.

Relative abundance of hares

The relative abundance of hares was estimated by the methods of spotlighting and faeces counts. Two methods were selected as increased relief, dense vegetation in unburned areas and burned vegetation may cause biases to census methods (Langbein et al. 1999). Burned and unburned areas with similar hunting statuses were compared, excluding by this way any influence of hunting. To minimize bias, spotlight and faeces counts were performed by the same researchers.

Spotlight count index

In the spotlight method, hares were counted from a 4-wheeled vehicle travelling at about 15-20 km/h on forest unpaved roads. Vegetation along roads was representative of that occurring across the broader landscape and burning along roads was not different as well. The crew consisted of the driver and two observers, each one holding two million candle spotlights which were used to light up both sides of the road. Surveys began two hours after sunset and were completed within three to four hours (Langbein et al. 1999). Visits were postponed during intensive rains and in thick mist. In Kassandra one visit was carried out each month in spring and summer and about two visits each month in autumn and winter, in total 30 visits were carried out in first and second years after wildfire. In Lagada six visits were carried out in summer of second year after the wildfire.

Additionally, as most of the hares were seen initially or were approached later at a distance of less than 30 meters, it could be distinguished which hares weighed more than 3 kg (developed hares) and which less than 2.5 kg (juvenile hares), that is younger than three months old (Bray et al. 2002). Hares which could not be seen well or appeared to weigh about 2.5-3 kg, were excluded from this distinction.

Belt transects for faeces counting

Belt transects for faeces counting were placed in sampling units along the length of the forest roads that were spotlighted. Sampling units were positioned every 600 m following the random selection of the initial sampling unit (Calvete et al. 2004). The belt transects were set up in the same sampling units each year. In each sampling unit four sections of belt transect of 100 m length and 0.5 m width were installed (Fa et al. 1999; Calvete et al. 2004; Murphy and Bowman 2007). Two sections were located on each side of the road at a constant distance of 30 m from the road, and two more sections were located at a distance of 50 m from the road. In cases where obstacles (logs, dense shrubs, steep slope) were encountered, researcher went around until the 100 meters of transect was completed. Consequently, faeces were counted at a total distance of 400 meters (4 sections × 100 m length) from each sampling unit.

The sections were positioned 30 to 50 meters from the road so as not to be influenced by negative factors (e.g. disturbances by cars or livestock), but not too far so that the data could be compared with that of the spotlight method. This method is thought to be more effective than setting small plots as it covers a larger surface area and therefore it is more likely to count hare faeces in an area with low hare density. In this way, one avoids zero values which would complicate the statistical analysis (Quinn and Keough 2002). The faeces counting method was carried out once a year, so that the decomposition of the previous year's faeces occurred in Mediterranean conditions (Palomares 2001). Faeces disappearance rates between burned and unburned areas are not expected to significantly differ. Three months after wildfire in Kassandra (November) the sampling method was tested by positioning five sampling units in the moderately burned area and five in the unburned area, later faeces counts were performed each April for six years. In Lagada, faeces counts were performed at the end of August once, at the second year after the wildfire.

Additional to this research, a number of hares were also kept in captivity and provided with herbaceous food. Counts of faeces size revealed that faeces of young leverets (until two months old) were usually smaller in size (smaller than the one half of size) than that of juveniles or adults. Therefore, the faeces counted in Kassandra Peninsula were classified into two sizes, large and small (Southgate 2005; Rouco et al. 2012), to examine any differences in the ratio of young leverets/older hares between burned and unburned areas. As small faeces we classified those that were smaller than the one half of size of large faeces.

Hare collection

In November 2007, one year after the wildfire in Kassandra, we requested and received permission from the Forest Service to collect hares from burned areas and adjacent unburned refuges (in both areas hunting was prohibited). Twenty two hares were captured in accordance with the guidelines for the use of wild mammals in research (Animal Care and Use Committee 1998). Moreover, the number of collected hares was kept as possible small due to the discontent that could be caused to the local community by capturing hares. Thus additional hares were collected by hunters in adjacent unburned areas during daylight. The animals were inspected (hares that were collected at night were also weighed) and their sex was determined. Each hare was dissected to obtain one eye, one front leg, the stomach, the liver, the kidney, and the uterus (only one female was pregnant, and found in moderately burned area). The eye was preserved in 10% formalin and the other organs were frozen at -20°C, five to eight hours following death.

To determine the age of each specimen, the crystalline lens was weighed according to Suchentrunk et al. (1991). Hares were divided into three age classes: leverets and juveniles with dried eye lens weights <235mgr, near adults with weight 235-279.9mgr, and adults with dried eye lens weights ≥280mgr (Suchentrunk et al. 1991). Hare body condition was determined using the kidney fat score on a scale 0-5 (Bonino and Bustos 1998; Banks et al. 1999, Jennings et al. 2006). Stomach contents were removed carefully so no residual stomach tissue remained, then were dried, weighed, and milled to 1 mm. Crude protein content was measured as Kjeldahl N × 6.25 (AOAC, 2006), and crude fat and fibre contents were determined according to AOAC (2006). According to Banks et al. (1999) and Hackländer et al. (2002), high crude fat and protein levels together with low crude fibre content, indicate a nutrition-rich diet. Therefore the ratio of crude protein and fat to crude fibre was used as an indicator of diet quality.

Livers were examined for the presence of lesions and the method of Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR) was then applied to detect European Brown Hare Syndrome virus (EBHSV). Additionally, 29 serum samples were tested using the Indirect Immunofluorescence Test (IFAT) for the detection of antibodies against *Francisella tularensis*, *Brucella* spp, and *Toxoplasma gondii*.

Fire influence

Fire influence (FI) was estimated around each sampling unit (where belt transects for faeces counting were set up) in the Kassandra site. A 600×600 m square (36 ha) was marked, with its centre on the sampling unit. These dimensions were selected so a high number of scent stations could be placed along the road and also to take into account the home range of hare (Homolka 1985; Reitz and Leonard 1994). The assumption was made that for any given 600×600 m square, the combination of more burned area and higher aggregation of the remaining forest and shrubby patches, leads to greater FI (Birtsas et al. 2012):

$$FI = (BV \times CA)^{1/2}$$

where BV represents the burned vegetation and it is the sum of the percentages of the completely burned forest area and half of the completely burned agricultural area (because the wildfire in the agricultural area was not as intense as in the forested area), and CA is an indicator of woody cover absence after the wildfire (see Birtsas et al. 2012 for a detailed description).

Statistical analyses

The number of spotlighted hares per km and per visit was compared using the parametric paired Student's t-test or the one-factor within subjects Analysis of Variance (ANOVA). Using this approach, the effect of factors that may affect the detection of hares between visits, such as moon phase and wind were excluded. When the assumptions of using the parametric tests were not met (few observations, non-normal distribution) the non-parametric tests of Wilcoxon and Friedman were used, respectively (Zar, 1996).

The numbers of faeces counted per belt transect (400 m) were compared using the independent samples t-test or one-factor between subjects ANOVA. In most cases data were transformed using the equation $\ln(x+1)$. When assumptions were not met the non-parametric tests of Mann-Whitney and Kruskal Wallis were used, and post hoc tests were performed using the Bonferroni test whenever the null hypothesis was rejected. At the micro-scale level, linear regression analysis was used to explore the relationships between the number of faeces/belt transect and fire influence parameters in the moderately burned area.

The parameters of diet quality and body condition were tested either by one or two factors (without interactions) between subjects ANOVA. The proportions of hare ages and faeces spotlighted hares sizes were compared using the Chi-square test and, where the expected values in any cells of the contingency table were below 5, with Fischer's exact test (Zar 1996). Normality was investigated using the Shapiro-Wilk method, and homogeneity of variances by Levene's test. Statistical analyses were performed using IBM SPSS 20.0. The level of significance was set to be α=0.05.

5.3 Results

Hare relative abundance in burned and unburned areas

Spotlight count index

A total of 30 visits to the Kassandra study area (from 23/10/2006-07/08/2008) identified 143 hares along a total distance of 837.8 km of forest roads (458.8 km in burned areas and 379 km in unburned areas). During the first year after the wildfire, the hares/km ratio was lower in burned areas than unburned. In the intensely burned area of Kassandra no hares were recorded until spring 2007. In Lagada six visits in summer of second year after the wildfire identified 35 hares along a total distance of 162 km of forest roads (90 km in burned areas and 72 km in unburned areas). In the second year the hares/km ratio was higher in the burned areas compared to non-burned in both the Kassandra and Lagada study areas (**Table 1**).

Table 1. Spotlight recorded hares in Kassandra and Lagada study areas.

		Season	*Not burned areas*			*Moderately burned areas*			*Intensely burned areas*		
			hares	km	hares/km	hares	km	hares/km	hares	km	hares/km
Kassandra Peninsula	1st year after wildfire	*Autumn*	3	25.5	**0.118**	3	28.5	**0.105**	0	13.5	**0**
		Winter	4	34	**0.118**	3	38	**0.079**	0	18	**0**
		Spring	6	34	**0.176**	3	38	**0.079**	0	18	**0**
		Summer	3	25.5	**0.118**	3	28.5	**0.105**	3	13.5	**0.222**
		Total	16	119	**0.134**	12	133	**0.090**	3	63	**0.048**
	2nd year after wildfire	*Autumn*	31	139	**0.223**	21	98.7	**0.213**	7	39.5	**0.177**
		Winter	1	37	**0.059**	5	19	**0.263**	1	9	**0.111**
		Spring	10	58.5	**0.171**	16	34.1	**0.469**	6	14.5	**0.414**
		Summer	3	25.5	**0.118**	7	28.5	**0.245**	4	19.5	**0.205**
		Total	45	260	**0.173**	49	180.3	**0.272**	18	82.5	**0.218**
		Total	**61**	**379**	**0.161**	**61**	**313.3**	**0.195**	**21**	**145.5**	**0.144**
Lagada 2nd year *Summer*			12	72	0.167	23	90	0.255			

In the first year following wildfire, the number of hares/km/visit did not differ between burned and unburned areas (paired t test, P=0.199). Comparing the three areas, the hares/km/visit was marginally not different (Friedman $x^2(2)=4.98$, $P=0.08$). The number of hares/km/visit was marginally lower in intensely burned compared to non-burned areas (P=0.071) (**Fig. 2**).

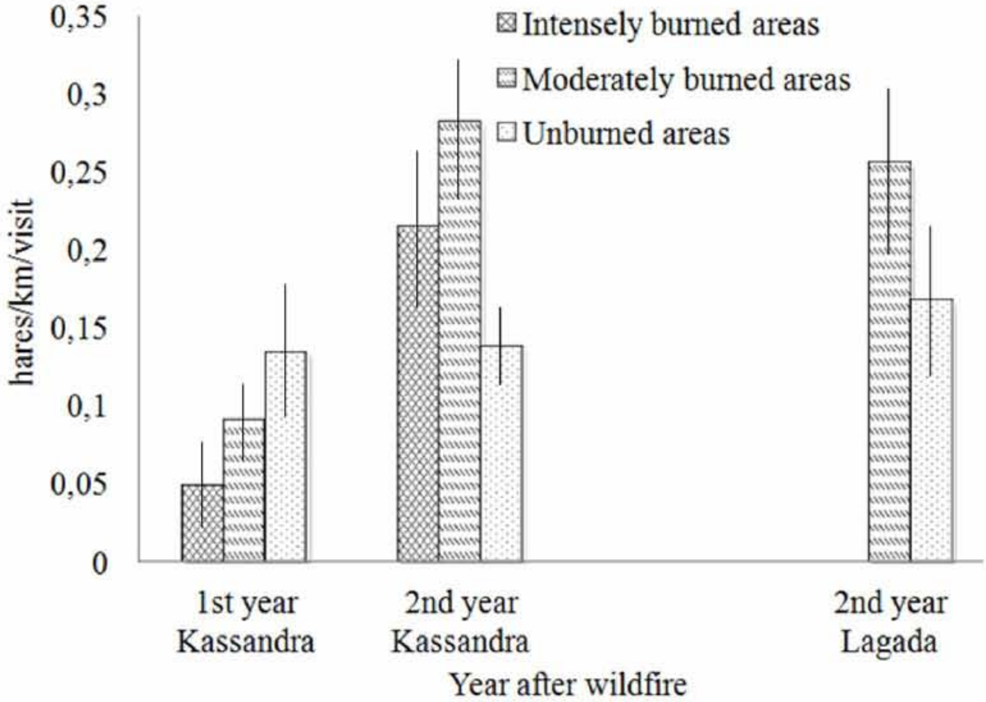

Fig. 2. *Mean ± SE of number of hares/per km/per visit during the first year (n=14 visits) and second year (n=16 visits) after the wildfire in Kassandra Peninsula, and the second year after wildfire in Lagada (n=6 visits).*

In the second year after the wildfire, the number of hares/km/visit was significantly higher in burned areas than in unburned refuge areas (paired t test, P=0.02). Comparing the three areas, the hares/km/visit is marginally different ($F_{2,30}=5.9$ P=0.046). The number of hares/km/visit was significantly higher in moderately burned compared to unburned areas (P=0.039) (**Fig. 2**). The study in Lagada showed similar results with more hares found in burned areas during the second year (paired t test, P=0.019).

Belt transects for faeces counting

In the first year following wildfire, the number of faeces/belt transect was significantly lower in burned areas compared to unburned (t test, P=0.008) (**Fig. 3**). Comparing the three areas significant differences were found ($F_{2,30}=3.887$, P=0.032). Post hoc comparison showed that this difference was significant between unburned and moderately burned areas (P=0.039), and also between unburned and intensely burned areas (P=0.022). No significant difference was found between moderately and intensely burned areas (P=1).

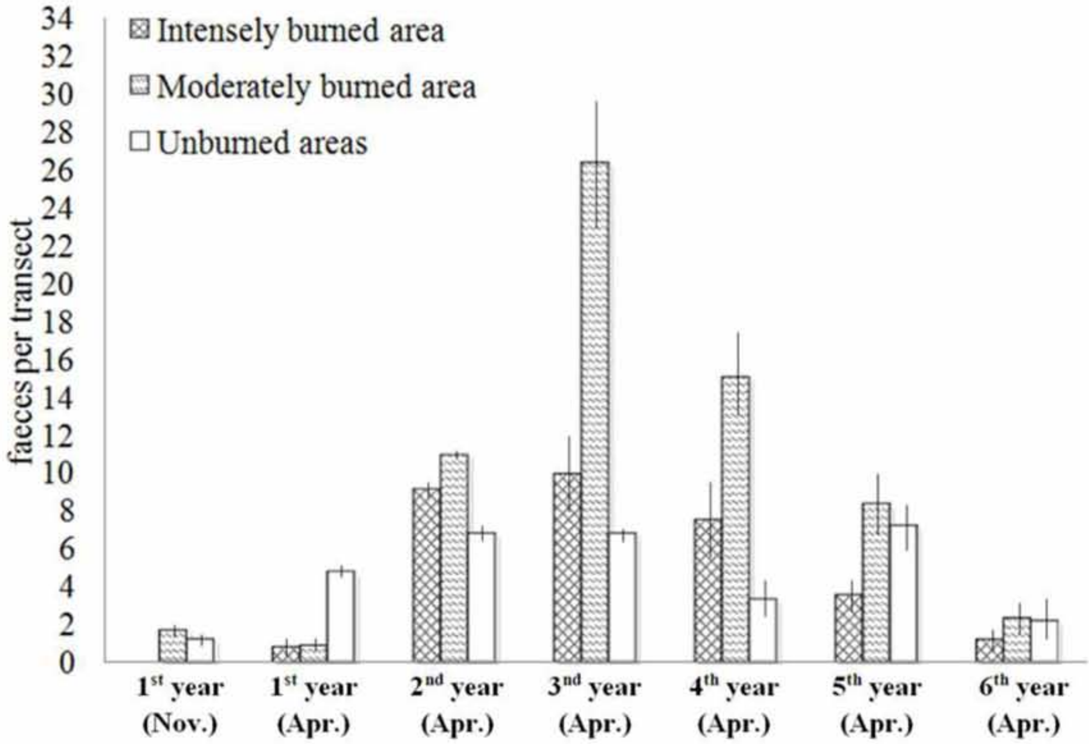

Fig. 3. *Mean ± SE of number of hare faeces per belt transect (400 m) in Kassandra Peninsula. Three months after wildfire (November) the sampling method was tested by positioning five transects in the moderately burned area and five in the unburned area. In subsequent visits, 7, 17 and 9-15 transects were taken in intensely, moderately and unburned areas, respectively.*

In the second year following wildfire, comparison was applied between the burned and adjacent unburned refuges. The number of faeces/belt transect was higher in burned areas, however this did not differ significantly (t test, P=0.374). No differences were found between the three areas ($F_{2,36}$=0.436, P=0.65). In the summer counts at Lagada site, we counted 25.48±4.02 faeces (mean±SE, n=21) in the moderately burned area and 13.39±2.49 faeces (mean±SE, n=18) in the unburned area. This difference was found to be significant (t test, P=0.019).

In the third and fourth years following wildfire, hunting prohibition was lifted in the burned areas and the number of faeces/belt transect was significantly higher in burned areas compared to unburned hunted areas (third year: t test, P=0.039 and fourth year: P=0.044). Comparing the three areas we found significant differences (third year: $F_{2,32}$=3.883, P=0.031

and fourth year: $F_{2,32}$=3.763, P=0.034). Post hoc comparison showed that these differences were significant between unburned and moderately burned areas (third year: P=0.035 and fourth year: P=0.039). Between unburned and intensely burned areas no significant difference was found (third year: P=1 and fourth year: P=1). Additionally, no significant difference was recorded between moderately and intensely burned areas (third year: P=0.296 and fourth year: P=0.297).

The number of faeces/belt transect did not differ between burned and unburned areas in the fifth (t test, P=0.281) or sixth (Mann-Whitney z=-0.82, P=0.412) year following the wildfire. No significant difference was detected when comparing the three areas in these years (fifth year: $F_{2,32}$=0.61, P=0.55 and sixth year: Kruskal Wallis Test $x^2(2)$=0.895, P<0.639).

Micro-scale level study

In the moderately burned area of Kassandra Peninsula, fire influence (FI) on vegetation was different between the 17 adjacent sampling units. The average FI on vegetation was 0.776 (range 0.38-1). In sampling units with high FI, the number of faeces was greater in both years (1^{st} year: R^2=0.42, P=0.005, 2^{nd} year: R^2=0.27, P=0.034). More specifically, this was found in the first year for the burned vegetation index (BV: R^2=0.28, P=0.03), and in both years for the absence of cover index (CA: 1^{st} year R^2=0.398, P=0.007, 2^{nd} year R^2=0.286, P=0.027). Moreover, in the unburned area near the moderately burned area of Kassandra we did not find an increased number of hares or their faeces **(Fig. 4)**.

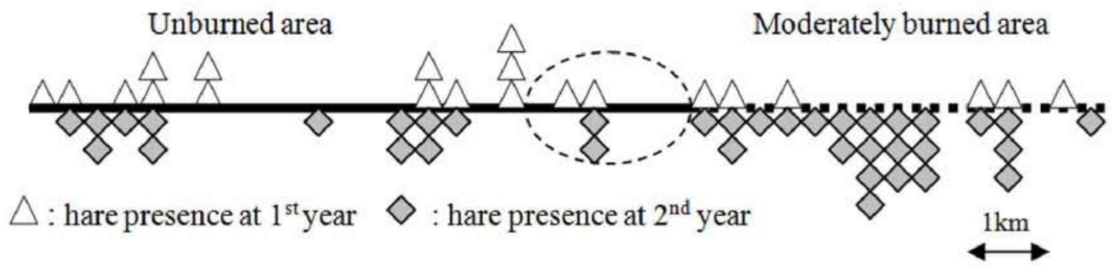

Fig. 4. *Depiction of positions of hare localisation between unburned (8.5 km) and moderately burned areas (5 km) in the first and second years following wildfire. (The dashed circle encompasses 2 km of unburned area adjacent to the burned).*

Age ratio

A total of 35 hares were collected in Kassandra. Eleven hares were collected in the burned area and eleven in the adjacent unburned areas. Thirteen more hares were collected in nearby unburned areas of Kassandra during the day. Age was determined in all hares. Adults were absent from the burned areas and the age ratio between burned (juveniles:6, near adults:5, adults:0) and unburned areas (juveniles:11, near adults:2, adults:11) was found to be significantly different (Fisher's test, P=0.007).

Regarding the ratio of small/large faeces no differences were detected between areas in the first (x^2 =1.175, P=0.278) and second year (x^2=0.675, P=0.411) following the wildfire. Similarly, according to the spotlight counts, the ratio of hares younger/older of three months old was not different between areas in the first (x^2=1.22, P=0.27) and second year (x^2=0.69, P=0.4) following the wildfire.

Body condition, diet quality and disease

Thirty-four kidneys and thirty-three stomachs were collected and analysed (two stomachs were rejected as one contained milk and the other blood). Analyses took into account hare age, sex and the time of day (day or night) they were collected. No differences were found in hare body condition between burned and unburned areas ($F_{1,18}$=0.169, P=0.685).

Body condition was not influenced by the dry weight of the stomach contents (R^2=0.06, P=0.182). Regarding diet quality, crude protein had a positive effect and crude fibre a negative effect on kidney fat, but these were not found to be significant (P>0.136). Fat in the diet favourably affected body condition, although this was marginally not significant (P>0.052). The diet quality indicator showed a positive correlation with body condition (R^2=243, P=0.0048). Between burned and unburned areas no significant difference was found (Fig. 5) between diet components and diet quality indicator ($F_{2,25}$<1.56, P>0.229).

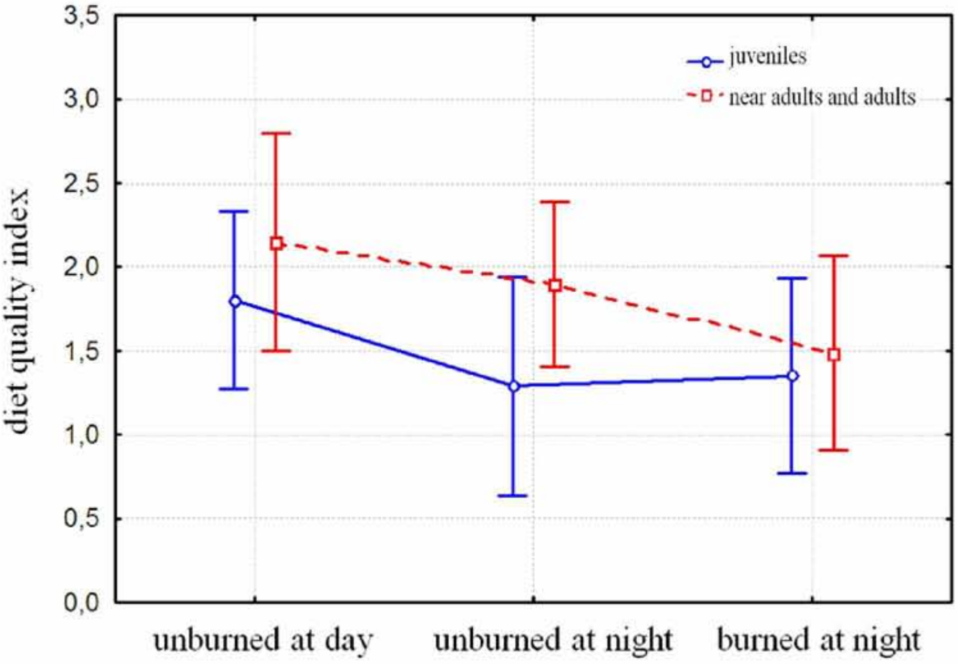

Fig. 5. *Means and confidence intervals (95%) for the diet quality index [(protein + fat)/fibre] for adult and juvenile hares collected day or night in burned and unburned areas.*

Using PCR, two hares were found to be positive to EBHS. One of these was found in the burned area and the other in the unburned area. This difference was not significant (Fisher's test, P=0.2). No antibodies were detected against any of the pathogens tested by IFAT.

5.4 Discussion

First year after wildfire

The wildfire in Kassandra was more severe in sites where cover by woody vegetation was higher. In contrast, the scattered agricultural fields in the forest contributed to the formation of unburned stands. These sites were located in moderately burned area and seem to be important for hare protection during and recover after wildfires. In the moderately burned area, hares were counted from the first visit to the study area (1.5 months after the wildfire). Keith and Surrendi (1971) did not find any population decline in moderately burned areas. However, in this study reduced relative abundance was observed in comparison with adjacent unburned areas, possibly due to the higher severity of the wildfire in Kassandra.

Increased numbers of hares in unburned area near the moderately burned area were not observed (Fig. 4), which supports the view that major population dispersal out of burned areas did not occur, or that hares soon returned to the burned areas after the wildfire. Keith and Surrendi (1971) also observed no dispersal from moderately burned areas to unburned areas. Hares avoid making home range movements of more than 1 km (Reitz and Leonard 1994), exhibiting home instinct (Jezierski 1968), whereas natal dispersal is less than 1.5-3 km for the majority of young hares (Reitz and Leonard 1994; Bray et al. 2007; Avril et al. 2011). Furthermore, after the rains of September (about one month after the wildfire) the herbaceous vegetation grew and could provide adequate nutritious food. It is probable that hares which moved from the burned areas, mainly the intensely burned area, returned to their pre-fire home ranges, however this requires further investigation.

In the intensely burned area of Kassandra, the first presence of hare was recorded eight months after the wildfire (Table 1). Similarly, Keith and Surrendi (1971) found no hares in intensely burned areas for one year after the wildfire. In the intensely burned area of Kassandra the hare population was likely to have been low before the wildfire due to the small numbers of forest openings and fields (Paci et al. 2007; Karmiris and Nastis 2007). Lower hare density was found in the intensely burned area in the following years. However, this does not seem to justify the total absence of hares for the first eight months after the wildfire. It is possible that in the intensely burned area the higher severity of wildfire caused mortality and a more permanent dispersal of hares, as adequate shelter and food was absent. Several researchers provide evidence of mortality by wildfires for *Lepus americanus* (Hakala et al. 1971) and *Sylvilagus floridanus* (Erwin and Stasiak 1979).

The intensely burned area is located within the moderately burned area and hare range expansion and any other interaction is presumably more probable between these two areas than between the intensely burned and the unburned area. Therefore, the study design used was not able to isolate any influence of adjacency. However, using the distinction of moderately and intensely burned areas, the differences due to wildfire influence were further investigated.

Second year after wildfire

The relations on hare abundance observed during the first year after the wildfire in Kassandra were reversed in the second year (Figs. 2 and 3). The spotlight counts showed more hares present in burned areas from autumn 2007, one year after the wildfire in Kassandra. Similar results were observed in the Lagada during the second year after the wildfire, where higher hare abundance was found inside burned rather than unburned area. Keith and Surrendi

(1971) report more hares in burned areas one year after wildfire. Other researchers also conclude lagomorph population increase at certain time periods after the occurrence of wildfire or prescribed fire (Gates and Eng 1984; Ferron and St-Laurent 2008; Rollan and Real 2010; Amacher et al. 2011)

One population can be higher than another because reproduction and immigration rates are higher and mortality and emigration rates are lower. Hares rarely disperse more than 1.5-3 km from their birth place (Reitz and Leonard 1994; Bray et al. 2007; Avril et al. 2011). Most hare abundance data for burned areas were collected at distances greater than 3 km from unburned areas, thus immigration was predicted to be low. Therefore, the most probable explanations for population increase in the second year are increased reproduction and reduced mortality rates. In burned areas of Kassandra we collected a higher number of juveniles and near adults than in unburned areas. This indicates that more neonates were produced and/or more juveniles survived.

Increase reproduction and survival rates could occur because of improved food quality and quantity (Hill 1972; Hackländer et al. 2002). The herbaceous biomass had increased considerably two years after the wildfire. However, in Kassandra study area in the autumn of 2007, about one year after the wildfire, the diet quality was not observed to differ between burned and unburned areas for all three components considered: crude protein, fat and fibre. Wildfires generally increase herbivore diet quality. This increase occurs immediately after the wildfire, but quickly decreases, and after an interval of one to two years there is no difference between burned and unburned areas (Hobbs and Spowart 1984; Carlson et al. 1993). The results of this study showed that the wildfire did not affect diet quality after one year. Moreover, body condition did not differ between hares of burned and unburned areas.

According to Sinclair et al. (1982), snowshoe hares require about 11% crude protein to maintain body weight. Studies in Central Europe found that the chemical composition of stomach contents is consisted of 19.43% crude protein, 4.14% crude fat and 15.36% crude fibre (Hackländer et al. 2002). In the present study, the diet quality was similar or better in our burned and unburned areas in comparison with Central Europe. Recorded values of mean crude protein were 26.7% in burned areas and 23.73% in unburned areas, of mean crude fat were 5.55% in burned areas and 9.1% in unburned areas and of mean crude fibre were 19.71% in burned areas and 19.68% in unburned areas.

Diet quality may have been higher in the first months after the wildfire, and thus the initially fewer hares in burned areas could have increased their reproduction rates. Hill (1972), states that the soil fertility increased the number of embryos of *Sylvilagus floridanus* by improving diet quality. However, in Kassandra, according to the faeces counting and spotlighting, the ratios of younger to older of two-three months old hares were not significantly different between areas in the first or second years after wildfire, thus indicating that the fecundity and production of neonates did not differ.

Lower mortality in burned compared to unburned areas may be due to lower predation, less diseases, and various anthropogenic causes such as hunting, poaching, agricultural practices and roads (Smith et al. 2004; Santilli and Galardi 2007). Anthropogenic influences did not change between the two years of research in the burned areas of Kassandra and Lagada. Additionally, the hunting influence was excluded as counting took place in adjacent wildlife refuges where hunting was prohibited.

Concerning disease, EBHS is one of the most important diseases for hare populations (Frölich and Lavazza 2008). Juveniles have lower susceptibility to EBHS (Frölich and Lavazza 2008), thus the influence of EBHS is expected to be lower in burned areas the first two years following wildfire when the population has a high percentage of young hares as this study found. However, no differences were detected for the infectious diseases examined and no disease symptoms were observed in collected hares. Wildfire seems not to influence EBHSV prevalence between burned and unburned areas one year after the wildfire. That year of 2007 is characterized by a low EBHS prevalence of 5.7% in all Kassandra Peninsula. Few years after the wildfire, in period 2010-12, the EBHSV prevalence was higher (28.6%) and also no difference was found between burned and unburned areas.

Predation depends on predation risk and predator pressure (Torre and Diaz 2004; Paci et al. 2007). Hares deposit their faeces in their feeding places (Litvaitis et al. 1985, Karmiris et al. 2010), and by counting faeces we found that hares prefer to feed in places with greater wildfire influence (FI). This holds mainly to the absence of woody vegetation (CA) and less to burned vegetation (BV). Therefore, hares preferred to feed in burned sites with more and larger openings, without woody vegetation. This has also been found in other studies on hare and reflects the lower predation risk in these sites compared to sites with woody vegetation (Paci et al. 2007; Karmiris and Nastis 2007; Karmiris et al. 2010). In southern Hellas two years after a wildfire, Karmiris et al. (2010) found that as the woody vegetation was decreasing due to burning, the number of hare faeces was increasing.

Trees provide perch sites for raptors and used by martens, so it is expected that the destruction of trees by wildfire will lead to the reduced presence of these predators (Torre and

Diaz 2004; Tapia et al. 2008). In addition, during the second year the rapid growth of herbaceous vegetation, together with the presence of branch soil-erosion barriers and branches from logging of burned trees, may have provided adequate and/or improved shelter to hares than before the wildfire.

Regarding predator pressure, stone marten was not detected in the burned areas during the second and third summers after wildfire in Kassandra (Birtsas et al. 2012) and fewer eagle owl (*Bubo bubo* L.) individuals were recorded during spotlight counts in burned than in unburned areas. However, Birtsas et al. (2012) found that during the second and third summers after Kassandra's wildfire, the red fox had higher visitation rates in scent stations positioned in the intensely burned area. A higher fox presence in the intensely burned area may contribute to the lower abundance of hares in intensely burned compared to moderately burned area.

Third to sixth year after wildfire

Two years after wildfire, the local Forest Service took the decision to lift the hunting ban enforced in burned areas of Kassandra due to the increased hare abundance in burned areas considering our study and the fact that shrubs had regained a considerable part of their pre-fire height. Following the hunting season, hare abundance was again higher in burned areas in the third and fourth years. Thus, hunting did not strongly influence the increased abundance of hares in burned areas even though more hunters visited these areas in comparison with unburned areas.

The research team visited the area accompanied by experienced local hare hunters to observe how the hounds behave in both burned and unburned areas. Observations showed that the initial detection of hares by hounds did not differ between burned and unburned areas. However, dogs had a higher difficulty when chasing hares after flushing, due to the impermeability of the thicker vegetation in burned areas at the height of dogs (numerous young shoots and fallen branches). According to discussions with seven experienced hunters, the hunting success does not seem to change in an unburned and a burned area after the second year from wildfire, however this requires additional study.

In the fifth and sixth years following wildfire, hare abundance does not differ between burned and unburned areas. This is expected as the woody vegetation creates again unfavourable conditions for hares covering the openings and increasing in height. Moreover, predator populations of species like stone marten may have recovered.

5.5 Conclusions and management implications

The relative abundance of hares was lower during the first year after wildfire in burned areas compared to unburned. Population abundance was even lower in the intensely burned area where wildfire severity was higher and the wildfire completely destroyed all woody vegetation. A reversed relation was found in the second year, when the hare population was more abundant in burned areas compared to unburned. Thus it is proved that hare is influenced by a wildfire; however has the ability to recover rapidly after this severe habitat disturbance. This increased abundance in burned areas was observed until the fourth year after the wildfire. In the fifth and sixth years no differences in abundance were found between burned and unburned areas.

Diet quality, body condition and diseases were not found to differ between burned and unburned areas one year after the wildfire. The reduced abundance of some predator species, the opening of forest vegetation, and the higher presence of juveniles and near-adults in the burned area with a stable presence of neonates, indicate lower predation in burned areas compared to unburned areas. Thus, predation seems to be an important limiting factor for hare populations and that wildfire disturbance decreases its impacts. In other words, habitat disturbance regime in Mediterranean ecosystems influences the predation regime on hare.

Additionally, post-fire forest treatments may influence predation risk on hares. Post-fire tree logging is the most commonly applied practice in burned forests. The dispersion of branches was not found to be beneficial for wild rabbit (*Oryctolagus cuniculus* Lilljeborg, 1873), but in contrast, their collection seems to provide valuable shelter (Rollan and Real, 2010). In our study area many branches were gathered to branch soil-erosion barriers, a practice which we believe that it provides cover to hares. A future study should research for a proper design for provisioning suitable cover to hares after wildfires.

Hare populations are a renewable natural resource important for the hunting economy (Zamora et al. 2009; Papaspyropoulos et al. 2014), and thus responsible agencies should monitor their populations after a wildfire using appropriate counting methods. Of the two methods used in this research, faeces counting was more economical than spotlighting (Newey et al. 2003). Additionally, the prohibition of hare hunting should be examined for the first year after wildfire in a moderately burned area or, until the second year for an intensely burned area. After one year, the population of hares in burned areas is greater than those of wildlife refuges, a fact that might increase the risk by some pathogens (Gortazar et al. 2006).

Moreover, before the hunting season starts, we propose the adaptation of hares by hunting dog training for a period of two to three months. In this way hares will become more capable at escaping during the hunting period thus providing a higher quality hunting experience.

The formation of dense scrublands and woodlands has led to a decrease of hares in mountainous areas of the Mediterranean (Delibes-Mateos et al. 2009). The main change after the wildfire was the change of vegetation structure (reduction of woody cover from 83% to 7% in Kassandra burned area). The results presented here appear to support the statement that land abandonment is one of the main factors explaining the current trend of many wildlife species decrease in the Mediterranean region (Sokos et al. 2012). Therefore, this study highlights the utility of prescribed burning, grazing, logging and farming within forests as tools for biodiversity conservation in Mediterranean ecosystems by reducing forest biomass.

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6. HARE SURVEYS FOR DISEASES: DO WEATHER AND MOON HAVE ANY INFLUENCE ON SPOTLIGHTING?

Abstract

Nocturnal spotlight survey is a common census technique for many mammal species. The influence of abiotic factors may be essential for the appropriate design of censuses, however this method has not been examined sufficiently in relation to abiotic factors such as weather conditions and the moon. We tested their influence for a commonly counted species, the brown hare (*Lepus europaeus*). We conducted spotlight surveys at certain night time and transects in three upland Mediterranean areas, at different times of the year, different moon phases and under a variety of weather conditions. Wind speed and direction had an influence on hare counts with stronger winds resulted in fewer juvenile hares being observed, and more hares were counted when wind was blowing from the hare towards the vehicle. No significant effects were found from the other factors probably due to the intense relief and thick vegetation of the studied areas.

6.1 Introduction

Spotlighting (spotlight count or night lighting) is widely used to survey mammals during night (Scott et al. 2005; Sunde and Jessen 2013). This is a favored method of estimating abundance due to its efficiency, repeatability and no necessity for animals capture (Langbein et al. 1999). However, numerous studies have shown that several factors can affect the spotlight counts of mammals. These include the animal's activity patterns and behaviour (Brown and Peinke 2007), visibility (Parkes 2001; Barnes and Tapper 1985), relief and amount of vegetation cover (Ralls and Eberhardt 1997; Langbein et al. 1999; Tizzani et al. 2013), observer experience (Sunde and Jessen 2013), time of night and moonlight (Langbein et al. 1999; Scott et al. 2005) and weather conditions (Langbein et al. 1999).

Spotlighting is a very common survey method for lagomorphs (Strauß et al. 2008; Tizzani et al. 2013) and studies reveal sources of variation in counts due to weather conditions and moon phase. Newman (1959) found that the number of cottontails (*Sylvilagus floridanus*) observed during early morning or late afternoon roadside counts was higher with increasing snow cover, frost, rain preceding the count, and decreasing temperature and wind speed. In contrast significantly fewer brown hares were spotlighted when the temperature fell below 15°C in Britain (Barnes and Tapper 1985). Heydon et al. (2000) found no influence of rain or visibility on brown hares counted in Britain. Fafarman and Whyte (1979) refer that cottontail spotlight counts tended to be higher when rain was falling during the count and lower when moon illumination was increasing. Wind direction had also an influence, but no other weather factor affected their counts. In an arid region of Jordan, moonlight had a varied effect on spotlight counts of cape hare (*Lepus capensis*), but with similar densities during full and no moon (Scott et al. 2005).

The above results seem to be contradictory for some factors and inconclusive for some others; one explanation may be the differences in abiotic and biotic conditions between geographical areas and differences between lagomorph species behaviour. Therefore, there is currently a difficulty in designing a study that takes weather and moonlight effects into account; consequently, their influence is generally overlooked in hare spotlight counts (Strauß et al. 2008; Sunde and Jessen 2013). In upland and shrubland ecosystems in particular we are unaware of any studies concerning the influence of abiotic factors on spotlighting brown hares. Thus, the objective of this study was to determine the influence of moonlight and weather conditions on the number of brown hares counted along spotlight transects.

6.2 Materials and Methods

Study area

The study was part of a larger research project on the influence of wildfires on brown hare that was carried out in the Kassandra Peninsula of Chalkidiki Prefecture, Macedonia, northern Hellas (Fig. 1). The Kassandra Peninsula has altitudes ranging 150-280 m above sea level, and the climate is Mediterranean with hot and dry summers, mild winters, and moderately rainy autumns and springs. Mean annual precipitation is 581 mm and the mean annual air temperature 16.3°C. The landscape has intense relief with steep slopes in some positions.

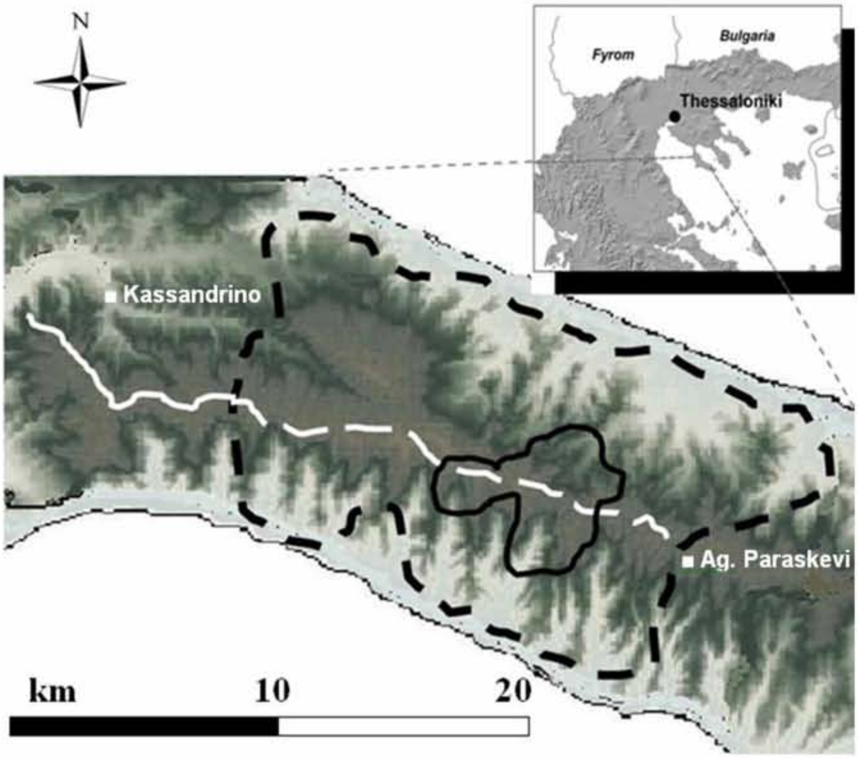

Fig. 1 *Location of the study area. The dashed black line indicates the moderately burned area and the solid black line denotes the intensely burned area. The solid white line shows the transect within the unburned area, and the dashed white line shows the two transects within the burned areas*

The study area is covered mainly by forest vegetation of Aleppo pine (*Pinus halepensis*) and broadleaved shrubs (*Quercus coccifera*, *Pistacia lentiscus*, *Phillyrea media*, *Cistus incanus*), while there are also scattered agricultural fields, mainly comprising olive groves, cereal and few vetch crops. Wildfire occurred in Kassandra on 21/8/2006 and burnt an area of 68.7 km². The woody plants regenerated quickly after wildfire and shrubs resprouts reached the height of about half a meter in the first year after the wildfire.

The spotlight transects stretch along the ridge of the peninsula and the unsurfaced forest roads. The transects had a total length of 22 km: 8.5 km passing through unburned areas, 9.5 km through moderately burned areas, and 4 km passing through intensely burned areas (Fig. 1).

Spotlight counts

Hares were spotlighted in the study area at different times of the year, under different weather conditions and moon phases, but along the same transects and at the same time of night and all transects were surveyed each night. To avoid observer bias, almost the same crew undertook surveys in each visit. Hares were counted from a 4-wheel truck traveling at about 15-20 km/h on forest roads. The crew consisted of the driver and two observers, each one holding two million candle spotlights which were used to light up both sides of the road. Surveys began two hours after sunset (Verheyden 1991; Langbein et al. 1999). Visits were postponed during intensive rains and in thick mist. About one visit was carried out each month in spring and summer and two visits in autumn and winter. The survey lasted for two years during the first and second year after wildfire, and the total number of visits was 30.

Additionally, as most of the hares were seen initially or were approached later at a distance of less than 30 meters, it could be distinguished which hares weighed more than 3 kg (developed hares) and which less than 2.5 kg (juvenile hares), that is younger than three months old (Bray et al. 2002). Those which could not be seen well or appeared intermediate in weight were not categorized.

Weather conditions and moon phase

According to the literature concerning influences on spotlight counts, we selected nine explanatory variables to describe the variable "hare counts", as shown in Table 1. During each visit, wind level and cloud cover were recorded. Available climatic data for the study area on each visit day were obtained from the nearest climatic station of the National Observatory of Athens (http://penteli.meteo.gr/meteosearch/). Precipitation values of the visit day and two days before, the mean air temperature, wind speed and direction were taken into consideration in all analyses (Table 1). Moonlight illumination values for each day were obtained from the US Naval Observatory (http://aa.usno.navy.mil/data/docs/MoonFraction.html). The level of moonlight was adjusted by multiplying illumination of the moon and cloud cover according to Brown and Peinke (2007). Cloud cover was categorized as 1=no cloud cover, 0.5=intermediate, 0.1=complete cloud cover. We used 0.1 instead of zero for complete cloud cover, because the darkest of the nights with a full-moon was brighter than any new moon nights, as the moonlight penetrated the clouds (Griffin et al. 2005).

Table 1 Weather and moon variables and their values during the counts

Variable	Meaning	Type	Mean ± SE	Min.	Max.
Moon	Degree of moon appearance during the visit	Scale	0.42±0.07	0	1
Clouds	Degree of cloud cover during the visit	Ordinal	1[a]	0.1	1
Moonlight	Interaction of moon and clouds during the visit	Scale	0.28±0.06	0	1
Temperature	Mean temperature on the day of the visit (°C)	Scale	13.4±1.55	0	33.8
Rain1	Precipitation during the day of visit (mm)	Scale	0.3±0.16	0	4
Rain2	Precipitation during the day of visit and one day before (mm)	Scale	2.11±1.03	0	23.6
Rain3	Precipitation during the day of visit and two days before (mm)	Scale	3.79±1.25	0	23.6

Windspeed	Mean wind speed on the day of the visit (km/h)	Scale	8.1±0.91	1	22.7
Windir	Direction of wind on the day of the visit	Circular	east[b]	-	-

[a]median

[b]the mode value of wind direction

Statistical analysis

The hares observed during each visit were divided into two categories, each one consisting of three dependent variables. The first category was relevant to the hares' age. It included the variable "juv" with the juvenile hares, the variable "dev" with the developed hares, and the variable "total" with the sum of counted hares. The second category was relevant to the study area. It included the variable "intBurned" with the observed hares/visit in the intensely burned areas, the variable "modBurned" with the observed hares/visit in the moderately burned areas, and the variable "unburned" with the observed hares/visit in the unburned areas. We also used an additional variable, "totBurned", which was the sum of "intBurned" and "modBurned" variables.

As shown in Table 1, our aim was to examine the influence of seven scales, one ordinal and one circular variable to the dependent variables. Circular variables have a special statistical analysis not suitable for the usual types of variables, and for this reason we transformed Windir into two variables sinWindir and cosWindir. That is, we followed the transformation suggested by Jammalamadaka and Lund (2006) by computing the sine and cosine of the wind angular measurement in order to use it in the typical statistical analysis of weather and moon data.

Firstly, descriptive statistical methods were used to analyze the data. We computed mean values, standard errors and median values of the variables. We then used the non-parametric Sign test. This test is robust to normality and homogeneity of variance and makes no assumptions about the original distributions of the variables (Gray and Kinnear 2012). The test was used to examine if there was a statistical significant trend in hare counts due to hare abundance changes and other factors.

When a statistical significant difference was confirmed, we removed the trend from the series of observations. This is a common procedure in time series analysis (Box et al. 2008). To determine the real influence of the weather and moon variables on the hare population, one has to remove any existing population trend. In the time series $\{y_t\}_{t=1}^{n}$, the trend can be removed if we create a new time series $\{x_t\}_{t=1}^{n-1}$ using the equation (Chatfield 2004): $x_t = y_t - y_{t-1}$. We used the Kendall tau-b correlation coefficient to relate the new series with the weather and moon variables. The analysis was performed using the statistical software IBM SPSS 20.0 and the significance level was set to $P<0.05$ and tending towards significance at $P<0.1$.

6.3 Results

A total of 30 visits to the study area identified 104 hares in a total distance of 660 km. During the counts, the moon was usually below crescent, but in four visits it was around full moon and cloud cover was absent (Table 1). Mean daily temperature varied from zero in winter to 33.8°C in summer. There was no snowfall and rainfall was low on the day of the count, although for the period of two days before the visit, rainfall >10mm was recorded in five visits. A light breeze blew during most counts with a maximum speed of 22.7 km/h (tree branches move).

The Sign test confirms that there is an increasing trend of observed hares in the second year after the wildfire in the intensely burned and the moderately burned areas of the study area (P = 0.016 and P = 0.004 respectively). The unburned areas show no trend (P = 1.0). Therefore, the procedure of differencing was applied in order to eliminate the trends. An example in Fig. 2 shows the result of differencing the trended series. The left Figure shows the initial total count of hares, where a trend is confirmed, and the right Figure shows the detrended series.

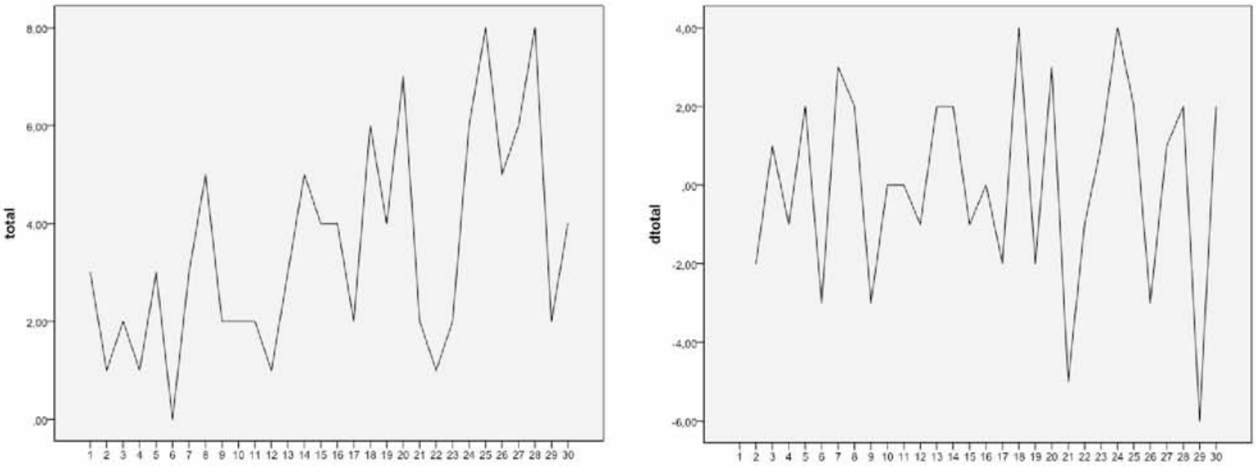

***Fig. 2** Initial (left) and detrended series (right) of hare counts during the 30 visits (total of counts from the three transects)*

The detrended series were checked for their bivariate correlations with the weather and moon variables. Table 2 shows that significance at the 10% level was found between wind speed and the counts of juvenile hares, showing a negative linear correlation. This means that stronger winds resulted in fewer juvenile hares being observed. This relationship is marked in bold in Table 2. Another marked relationship, the one between intensively burned areas and the sine of Wind Direction (sinWD), indicates that east winds are coincided with an increased number of recorded hares.

Table 2 Kendal's tau-b correlation coefficient of counted hares and weather and moon variables. The bold numbers mark significant relationships at the P<0.1 significance level

Variable	moon	clouds	illumin	temp	rain1	rain2	rain3	windspeed	sinWD	cosWD
juv	0.163	0.087	0.106	0.124	-0.136	-0.046	-0.044	**-0.267**	0.057	0.109
P	0.252	0.597	0.453	0.378	0.404	0.772	0.775	**0.058**	0.705	0.474
dev	-0.048	-0.024	0.005	-0.010	-0.151	0.092	0.045	0.205	-0.071	0.009
P	0.727	0.880	0.971	0.942	0.344	0.555	0.765	0.138	0.636	0.951
total	-0.115	-0.063	-0.144	0.026	-0.161	-0.158	-0.006	0.026	0.202	0.032
P	0.403	0.690	0.295	0.849	0.306	0.310	0.967	0.849	0.172	0.831
intBurned	-0.194	-0.012	-0.215	-0.041	0.102	-0.158	0.127	0.163	**0.300**	0.047
P	0.184	0.943	0.141	0.775	0.544	0.340	0.420	0.261	**0.057**	0.765
modBurned	-0.115	-0.080	-0.085	0.057	-0.110	-0.214	-0.023	-0.076	-0.093	-0.205
P	0.415	0.618	0.547	0.684	0.492	0.178	0.881	0.587	0.539	0.174
totBurned	-0.153	-0.050	-0.166	0.035	-0.048	-0.206	0.067	0.013	-0.019	-0.188
P	0.274	0.754	0.234	0.803	0.765	0.191	0.658	0.924	0.897	0.210
unburn	0.097	0.119	0.074	-0.019	-0.186	0.148	0.052	-0.036	0.131	0.197
P	0.504	0.474	0.607	0.894	0.263	0.364	0.740	0.804	0.395	0.202

juv: juvenile hares, dev: developed hares, total: sum of juvenile and developed hares, intBurned: total hares in the intensely burned areas, modBurned : total hares in the moderately burned areas, unburned: total hares in the unburned areas, totBurned: sum of intBurned and modBurned

6.4 Discussion

Analyses on spotlight counts showed that wind speed and wind direction influenced counted hares in some cases. Similarly, Newman (1959) found that the number of cottontails observed during roadside counts was higher with decreasing wind speed. Wind causes the moving of vegetation and thus the ability of observer to detect the hares and especially the smaller juveniles appears to be decreased. Moreover the movable of vegetation may cause fear to juveniles and thus the decreasing of their movements. High speed winds have been associated with decreased activity in lagomorphs (Lechleitner 1958; Smith 1990; Villafuerte et al. 1993).

From the other side, the movement and the noise by the wind-blown vegetation apparently make the detection of the approaching vehicle more difficult for hares and thus hares may stay nearer to the road and thereby the probability of be counted is increased.

Perhaps this is why a positive relationship was found, although not significant, between the intensity of the wind and the recorded developed hares, which as more experienced than juveniles may know that should go away when a car is approaching and this was observed during this study. Other studies have also found that the distance at which a mammal move away from an approaching human is negatively correlated with wind speed (Karlsson et al. 2007). Moreover, wind direction was also significantly associated with hares counted in intensely burned area, which tended to increase when the wind was blowing from the east direction, this means from the hare to vehicle (Fig. 1). The east wind appears to take away the noise under the circumstances of intensely burned area with a straighter west-east road than in other areas.

Analyses on spotlight counts indicated that no other weather or moon variable had an impact on the counted hares. Precipitation variation was relatively small and rainfall in the day of visit was below 2.5mm, under these conditions none important influence of rains was detected. These conditions provided us the opportunity to better examine the influences of temperature and moonlight. However, none appeared to have any effect on hare counts.

In our study area the steeply upland area and the thick vegetation do not permit the observation of hares at long distances as in open areas, thus in upland landscapes, such as the Kassandra Peninsula, the counting of hares seems to depend more on randomness: that is if a hare is close to the road at the time the car passes. Nevertheless, in flat landscapes hares can be observed at greater distances. In this case the observer's ability and hare's behaviour may have a higher importance for the detection, and may be more influenced by weather and moon. This may explain why in Britain it was proved that temperature reduced brown hare activity, with significantly fewer hares spotlighted when the temperature fell under 15°C (Barnes and Tapper 1985). For other lagomorph species, Newman (1959) found that the number of cottontails counted increased with the decrease of temperature, and Rogowitz (1997) found that temperature had no significant effect on movements of white-tailed jackrabbits (*Lepus townsendii*).

Many nocturnal mammals react to increasing moonlight by restricting their movements and the use of open areas to avoid detection by predators (Kolb 1992; Beier 2006). In Ireland, moonlight negatively affected counts of Irish hare (*Lepus timidus hibernicus*) during winter in areas where the vegetation is usually short (Reid et al. 2007). The same was found for white-tailed jackrabbit that reduces its activity during periods of greater moonlight in grasslands (Rogowitz 1997).

In our forested study area, the vegetation, even in burned areas a few months following the wildfire, provided adequate cover for hare, a fact that may decrease the detection probability by predators and the moonlight influence. A similar case may be that of snowshoe hares (*Lepus americanus*) which are less active in open areas during nights with increased moonlight in winter (Gilbert and Boutin 1991; Griffin et al. 2005), but this was not found in the snow-free season when the vegetation cover and hiding opportunities were higher (Griffin et al. 2005). In contrast, black-tailed jackrabbits in northern Utah were more active when moonlight was more intensive during fall, winter and spring. During summer moonlight did not appear to influence jackrabbit activity (Smith 1990).

In conclusion, only wind speed and direction had an influence on hare counts tending towards significance. Thus, the researcher should expect to see fewer juvenile hares in windy days, something that would not apply to developed hares. Yet if the wind is blowing from the hares towards vehicle the researcher is likely to count more hares. Temperature and moon did not influence spotlight counts. Precipitation also had no effect possibly due to its low variability in this study. In a future study, will be a need to test the method in relation to weather and moon in different ecotopes, especially concerning relief and vegetation structure.

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7. CONSERVATION CONSIDERATIONS FOR A MANAGEMENT MEASURE: AN INTEGRATED APPROACH TO HARE REARING AND RELEASE

Abstract

Wildlife managers are challenged with the task of deciding whether a management measure is appropriate or not, and furthermore they have to convince others about the merits of their decision. Population decline of some hare species (genus *Lepus*) has resulted in their Red Listing and conservation measures are being undertaken. Release or restocking is a frequent measure in some countries, and thousands of hares are released annually, mainly for hunting purposes. These hares can be obtained by either intensive or extensive rearing or translocation of the wild animals. Each method entails difficulties and different survival rates in the wild. Survival rates in the wild are low for hares intensively reared in cages but are higher for hares reared extensively in enclosures and wild translocated hares. The benefits of the hare release practice are significantly lower than the action's implementation cost. Hare releases have not increased significantly the wild hare population or the hunting harvest in areas where the practice has been applied. The risk of genetic and evolutionary degradation and pathogen transmission is possible in wild populations. The need for wise management of this practice is evident and the term 'Permitted Transferring Units' should be introduced to denote regions where hares should not be transferred for rearing and release.

7.1 Introduction

In practical wildlife conservation, managers are usually not well-supported by science or funding for decision-making, and frequently this leads to development and acceptance of dogma and wrong decisions (Sutherland 2000; Pullin et al. 2004). Animal releases and translocations are a controversial subject in wildlife conservation. These practices seem to enthuse some who consider it an easy option to increase animal populations. In contrast, others focus on the impacts of releases on wild populations (Sokos et al. 2008; Champagnon et al. 2012). Moreover, many farmers view wild animal rearing as an animal production enterprise whose products can augment wildlife populations (Tume 2000; Sánchez-García et al. 2012).

In the case of hare species, there is a long tradition of rearing and releasing that dates back to ancient times (Masseti and Marini 2008). Initially, translocated wild animals were mainly used for releases (Mazurkiewicz 1968; Schultz 1980; Masseti and Marini 2008). Today there is a high hunting demand for hares, and many responsible agencies are interested in hare species releases and translocations (Sokos et al. 2003; Santilli and Galardi 2006; Fischer 2011). However, eight hare species belong to a conservation category (Smith 2008), some due to their restricted geographic range (e.g. *L. castroviejoi*, Vulnerable), and some due to habitat degradation and competition (e.g. *L. corsicanus*, Near Endangered) (Smith 2008). Other hare species have low population densities, and in some countries are red listed, such as the brown hare (*L. europaeus*) in Britain, Austria and Switzerland (Smith et al. 2004).

Although the practice of hare release presently consumes a fair amount of economic resources in some countries, no attempt has been made to synthesize available knowledge of current hare rearing and release practices. This review aims: a) to explore the past and current state of hare releases in Europe, b) to compare the different techniques of hare rearing and release with respect to animal survival and cost, c) to highlight conservation issues of this practice, and d) to provide guidance on hare release management, as gaps exist in current legislation and consequences on wild hare populations have been found in many studies.

This systematic review was performed according to Littell (2008). Following the recommendation of Tranfield et al. (2003) that searches should not be restricted to bibliographic databases (Web of Science, Google Scholar and Agricola), we also used Google to identify unpublished studies, conference proceedings and other relevant scientific publications. Some additional publications were also found from reference lists and personal communication with researchers. The following keywords were used for these searches: "*Lepus*", "release", "restocking", "augmentation", "rearing", "breeding", "translocation", "re-introduction", "ripopolamento", "allevamento", "repeuplement", "lacher", "elevage", "repoblacion", "εκτροφή", "απελευθέρωση".

7.2 Hare releases in Europe

Hare releases are mainly practiced in southern and eastern Europe (Dematteis and Tizzani 2005; Misiorowska and Wasilewski 2012), whereas in some other countries such as The Netherlands, they are illegal (Rijks et al. 2012). In France, Italy and Hellas (Greece) there are many companies and private farms that advertise online the sale of reared hares for release into the wild. However, few data are available on the number of hares released each year, and in many countries there are no official statistics on these releases (ONCFS 2014; personal communication with experts). In the 1980s, about 40,000 brown hares were imported annually into France and another 100,000-120,000 into Italy from eastern Europe and other continents (Dematteis and Tizzani 2005; ONCFS 2014). The production of reared and released hares reached 200,000 animals per year in France (ONCFS 2014) and almost as many in Italy (Dematteis and Tizzani 2005). The annual production of reared hares in Spain (Sánchez-García et al. 2012) and Hellas is about 1000 individuals annually, whereas several hundreds of hares are released in other southern and eastern European countries (unpublished data, personal communication with experts). The importation of hares into France and Italy has declined, but still continues (Gaydou and Giovo 2009; ONCFS 2014).

In Hellas, hare releases were applied widely in the 1990s. However, in 2000 the responsible Hellenic Forest Service objected to the appropriateness of releases due to genetic introgression problems and low survival, and the practice was restricted for several years. From 2009 onwards the hare release practice resumed and the procedure was reorganized. It can now be approved if some, not well-defined, obligations on management, genetic and medical issues are met (a typical feasibility study and a substandard control of few animals); thus many weaknesses have arisen in procedure (Sokos et al. 2013). Today, about 1000 brown hares are released annually. These animals are mainly reared in cages in private farms and are sold to hunting organizations, transferring the animals to different geographical regions of the country. In addition, several hundred hares are kept in outdoor enclosures by amateur breeders and hunting organizations (Sokos et al. 2013).

7.3 Sources of released hares

Released hares derive from translocation of wild hares or are produced in captive rearing. Rearing of hares can be more or less artificial, more or less intensive – a process that is mainly dependent on the use of cages, small buildings or outdoor enclosures (Fiechter et al. 1988a).

In intensive rearing, captive stock is kept in small cages or occasionally buildings. The produced leverets remain in cages until their release or they are transferred into outdoor enclosures before release to assist their adaptation to the natural environment. Intensive rearing is used more frequently because it is profitable (Tomasevic 1977, Lainas 2006). In controlled cages farmers can use antibiotics and pesticides, and select stock animals with calm behavior (Tomasevic 1977; Tacconi et al. 1995).

In cases of extensive hare rearing, the breeding stock lives in outdoor enclosures usually of a few hectares in area. The production of hares in these enclosures is low, 2-6 hares/ha/year, as recorded in approximately 15 enclosures for five years in Italy (Santilli et al. 2004). Moreover, after a period of two to four years, episodes of high mortality have been recorded. Mortality occurs due to the density of the animals and the easier spread of parasitic diseases such as coccidiosis and strongilosis (Tacconi et al. 1995; Santilli 2006). Density control by removing a number of hares from the enclosures can decrease health problems (Santilli et al. 2004).

In cases of translocation, the capture methods used include netting, box trapping, snaring and night-lighting. In many cases trapping success is low (Nodari et al. 2005). Netting is used more in open landscapes where flushing of hares is easy (Takacs et al. 2009), whereas the other methods are used mainly in areas with thick vegetation (Bray and Leonard 2000). Bray and Leonard (2000) refer a capture efficiency of 0.19-0.43 trapped hares/trapper hour. Relatively low mortality of captured hares is caused by box trapping (up to 3%), night-lighting (up to 3%), and netting (up to 10%) (Griffith and Evans 1970; Abildgard et al. 1972; Cushwa and Burnham 1974; Bray and Leonard 2000). The above mortality rates are much lower than the 20-60% rate caused by snares with lock nuts (Douglas 1970).

Bisi et al. (2011), found that mountain hares (*L. timidus*) caught in baited cage traps were lighter than individuals caught using long nets, thus suggesting that the body condition of hares differs among the trapping methods, which may influence the subsequent survival (Sievert and Keith 1985; Marboutin et al. 1990). In addition, handling and transportation cause stress, weight loss and injuries that decrease survival after translocation (Mazurkiewicz 1968; Schultz 1980; Sievert and Keith 1985; Paci et al. 2006; Takacs et al. 2009). Tranquilization (Pasolini et al. 2013) and use of an obscuring cloth (Paci et al. 2012) may provide solutions to the stress parameter.

7.4 Comparisons of different techniques after release

About 60-90% of cage-reared hares die during the first 30 days after release (Table 1). The monthly survival rate of wild hares is 0.85-0.95 compared to ca. 0.2 of cage-reared hares (Lemnell and Lindlof 1982; Marboutin and Peroux 1995). The main cause of death is predation (Marboutin et al. 1990; Angelici et al. 2000). Abnormal behavior and physical weakness are the main reasons for high predation (Lemnell and Lindlof 1982; Pépin and Cargnelutti 1987, Meriggi et al. 2001).

The adaptation of cage-reared hares in outdoor enclosures before their release is believed to reduce some problems caused by cage-rearing. Lemnell and Lindlof (1982) and Fiechter et al. (1988a), did not observe any increase in the survival rates of adapted cage-reared mountain and brown hares, respectively. However, Dematteis et al. (2003) found improved survival rates for adapted brown hare juveniles only (Table 1). A even higher survival rate was recorded by Meineri et al. (1998), where 70% of the released brown hares were still alive six months after release. The animals were reared in cages and adapted in an enclosure of 1.500 m^2 located within the release area. However, hare predators were absent from the release area.

Hares which are born and reared in outdoor enclosures may have higher survival than cage-reared hares, as observed by Fiechter et al. (1988a) who refer to a higher hunter recovery rate for brown hares produced in enclosures. Misiorowska and Wasilewski (2012) were the first to study the survival of radio-tracked brown hares reared in enclosures. They recorded a survival rate of 57% in the first three months after release, and 45% at the end of the first five months. These results indicate a better adjustment to the wild of enclosure-reared hares compared to cage-reared ones.

Table 1. Total mortality (%) of radio-tracked hares (brown hares in most cases) at specific time intervals following release. Daily mortality after release is given by the equation: DM=1-[(1-TM)^(1/days)], where DM is daily mortality and TM is total mortality as a decimal (Heisey and Fuller 1985). Mortality is higher in the first days following release; therefore the comparison between the studies is not correct when time intervals are different

	Source of hares and time of release	Number and age of hares	Days after release	Total mortal. (%)	Daily mortal. (%)	Reference
Cage-reared	April–August	114 juveniles	7	50	9.4	Fiechter 1988
			42	79	3.6	
	May–August	102 juveniles	30	62.5	3.2	Marboutin et al. 1990
	throughout the year	46 adults and juveniles	30	82	5.5	Lemnell and Lindlof 1982
	throughout the year	44 adults and juveniles	30	78.95	5.1	Angelici et al. 2000
	June	30 juveniles	90	99.3	5.4	Meriggi et al. 2001
	throughout the year	25 adults and juv.	14	43	3.9	Pépin and Cargnelutti 1985
	Jan.–Feb.	25 adults and juveniles	210	100	2,2	Gatti et al. 1997
	February	20 adults	180	85	1	Gaydou and Giovo 2008
	April–June	16 adults (10-12 months old)	30	87.5	6.7	Karmiris 2006
			60	100	7.4	
	throughout the year	15 adults	10	60	8.7	Angelici et al. 1993
			90	67	1.2	
	July	10 juveniles	90	50*	0.77	Zanni et al. 1988
Cage-reared with adaptation	August	14 adults and juveniles	30	82	5.5	Lemnell and Lindlof 1982
	August	12 juveniles (70-110 days old)	15	25	1.9	Dematteis et al. 2003
			180	64	0.56	
	December	10 adults and 2 juveniles	15	91.7	15.3	Dematteis et al. 2003
			65	100	6.8	
	-	10	180	30*	0.2	Meineri et al. 1998
Reared in a 20 ha enclosure and released in November		60 (>6 months)	365	63	0.27	Misiorowska and Wasilewski 2012
Translocated	Jan.-April	75 adults and juveniles	16	83	10.5	Schultz 1980
	December	30 adults and juveniles	240	56.7	0.3	Meriggi et al. 2001
	January	20 adults	30	75	4.5	Zilio et al. 1997
	imported in December	26 adults and juveniles	240	70	0.5	Meriggi et al. 2001
	imported in February	18 adults	180	72	0.7	Gaydou and Giovo 2009
	May-August	17	30	37.6	1.5	Marboutin et al. 1990
	imported in Jan.–Feb.	19	210	84.2	0.9	Gatti et al. 1997
	Jan.–Feb.	14	210	78.6	0.7	Gatti et al. 1997
	imported in December	12 adults	15	50	4.5	Demmateis et al. 2003
			180	68	0.6	
	December	12 adults	15	41.7	3.5	Demmateis et al. 2003
			180	91.5	1.4	

* release areas without important hare predators

The translocation of wild hares is expected to give even higher survival rates as these animals were born in the wild. Table 1 shows that daily mortality at six to eight months is 0.5-1.4% for translocated hares compared to 1-2.2% for cage-reared hares. Meriggi et al. (2001)

examined the survival of brown hares from three different sources (cage-reared, translocated from another European country, and translocated from an adjacent area). Locally translocated hares showed the highest survival rates of between 40% and 50% at eight months following release (Meriggi et al. 2001). Gaydou and Giovo (2009) and Dematteis et al. (2003) also recorded survival rates of 28-32% at six months after release. However, studies of the snowshoe hare (*Lepus americanus*) show much lower survival rates (Schultz 1980; Sievert and Keith 1985). The usual mortality of translocated hares is at least double that of non-translocated wild hares (Table 1, Marboutin and Aebischer 1996).

Mazurkiewicz (1968) found that running performance of wild brown hares kept in transport cages decreased by half after 14 days. Antipredator behavior is important in lagomorphs (Rohner and Krebs 1996), and translocated hares have weaknesses in their new habitat, which increase the risk of mortality by predators or by other factors (Zilio et al. 1997; Jezierski 1967; Jezierski 1968, Ricci et al. 1983; Ferretti et al. 2010). The adaptation of translocated hares in enclosures within the release area does not increase the survival rates of translocated hares (Pielowski 1976; Schultz 1980; Fiechter 1988a; Benmergui et al. 1990).

7.5 Impacts of hare releases

Rearing and release can impact wild populations by (Champagnon et al. 2012): a) genetic degradation and evolutionary changes of breeding and wild stock; b) influences on demography and predation; c) increase of prevalent infectious diseases and parasites; d) neglect of other management measures which could be more effective.

Genetic degradation and evolutionary changes

Rearing and genetics

The genetic processes with the greatest potential impact on reared animals are inbreeding, genetic drift and selection (Snyder et al. 1996; Price 1999; McDougall et al. 2006). Whereas inbreeding and genetic drift result in loss of heterozygosity and produce random changes in gene frequencies, the changes resulting from selection are directional (Price 1999). According to Price (1999), there are three mechanisms of selection in captivity: a) artificial selection by the farmer, b) selection due to the captive environment, and c) relaxation of natural selection in the wild. Rearing that ignores selection influences in captivity, leading the captive population towards domestication (Snyder et al. 1996; McDougall et al. 2006). In the case of cage-reared hares, farmers usually prefer breeding stock that is less fearful to humans, exhibits less tendency to escape, and produces more leverets (Tomasevic 1977; Lemnell and Lindlof 1982; Bagliacca et al. 1992; Mantovani et al. 1993).

Reared mammals show a reduced ability to escape from predators, to obtain their food in the wild, to reproduce, and to have proper maternal behavior (Ford 2002, McPhee 2003; McDougall et al. 2006; Champagnon et al. 2012). In the wild, reared hares have low survival rates (Table 1), and Angelici et al. (2000) report no reproduction in six cage-reared female brown hares that survived in the wild for 75-284 days.

Robert (2009) notes that if the duration of a captive breeding program exceeds a certain threshold of 10–15 generations, it is likely that the genetic cost will be significant. In hare farms the number of wild hares brought into captivity is small and the same animals and their offspring are used for many generations. Thus, inbreeding (Hartl et al. 1992; Oyler-McCance and Leberg 2005) and mutations (Smith et al. 2010) may take place. Mamuris et al. (2001) found that reared brown hare populations showed little genetic differentiation in Hellas, and Smith et al. (2010) found male infertility which was caused after the admixture of captive brown hare populations.

Another problem is that in c. 1-2 m^2 rearing cages, hare leverets grow close to their mothers. This is in contrast with the behavior of wild hares where the doe leaves her neonates soon after birth (Broekhuizen and Maaskamp 1980). The high mortality of neonates in cages due to trampling or killing by their parents is a problem for hare farmers (Lainas 2006). Moreover, adult hares are usually solitary and the relationship between the two sexes in the wild is characterized by fights comprised of exchanges of blows with the front legs (Flux 2009). In many cases of cage rearing, the males and females live together. This can cause a state of stress in the females that can have consequences on reproduction, and sterility is very common in reared does (Martinet and Perretant-Langlois 1980; Pignatelli 1984). Therefore, apart from the selection forces caused by the human-modified environment in cages, animal welfare issues arise due to the poor well-being of caged hares (Mason 2010).

Release and genetic introgression

The exchange of captive hares of different origins between farmers and amateur breeders is common practice and is also applied in hare rearing. Admixture of captive populations and the release of allochthonous individuals may cause genetic introgression in indigenous hare populations (Andersson et al. 1999; Pietri et al. 2011). Before the introduction of brown hares in Sweden, the mountain hare occurred in the south of the country. However, following releases of brown hares, the mountain hare was extirpated from this area and hybridized with brown hare in other areas (Andersson et al. 1999). Pietri et al. (2011) found three hybrid specimens of apennine hare (*Lepus corsicanus*) in 67 individuals collected in Corsica, and notes that strict prohibition of releases of other hare species should be enforced.

In the 1990s, reared hares in Hellas were usually bought from private hare farms; however, the breeding stock had been previously imported from abroad. When these animals are released genetic introgression occurs (Stamatis et al. 2007). Hellenic brown hare populations are genetically different from central European and Italian populations, having higher mtDNA variability and geographical substructuring (Mamuris et al. 2001; Suchentrunk et al. 2003; Kasapidis et al. 2005). These studies contributed to justifications for the restriction of hare releases in order to protect this genetic diversity. However, Stamatis et al. (2007) found that genes from central and western Europe have survived in Hellas, as these genes were found in hares three years after release restrictions were enforced. However, these restrictions were not fully applied, and it is assumed that some hunting clubs or amateurs continued to release hares of unknown origin.

In Italy, the genetic differences found by mitochondrial DNA sequencing between brown hares of different altitudes correspond to differences in reproduction, such as the different number of leverets per pair, and different starting date and length of the breeding season (Ragagli et al. 2008). Therefore, gene introgression caused by releases of hares originating from different environments can possibly cause maladaptation in the local wild population.

Influences on demography and predation

Releases can affect demography by temporarily increasing population density, but this, in turn, may cause a decrease in survival and reproductive success of indigenous conspecifics as a result of density-dependent factors (Champagnon et al. 2012). Some studies have reported an inverse relationship between fertility and density in brown hares (Frylestam 1980) and between density and production of leverets (Angerbjörn 1986). Moreover, releases may attract predators and enhance their ability to catch hares (Costantini et al. 2005). Delibes-Mateos et al. (2008) found that when rabbit (*Oryctolagus cuniculus*) abundance increases, foxes (*Vulpes vulpes*) respond by increasing their predation on rabbits.

Increased prevalence of disease and parasites: a case study in Macedonia with EBHS

Hare releases can introduce pathogens into wild populations (Champagnon et al. 2012). European Brown Hare Syndrome (EBHS) was not reported to cause losses in Switzerland until 2000, and one explanation was the prohibition of importation of hares for release for more than 10 years (Frölich et al. 2001). In a study conducted in northern Hellas during 2007-2011, twelve of twenty hares which had 'Central European' mtDNA haplotypes, were positive for EBHSV. On the contrary, 33 hares which had 'typical' native Hellenic mtDNA haplotypes, were all negative for EBHSV. Interestingly, three of the twelve EBHS viruses isolated from hares which had haplotypes similar to those of imported captive-bred hares, were more closely related to the central European EBHS viruses than to other Hellenic viruses. Moreover, these three isolates were localized in the northern region of Chalkidiki. Therefore, alien strains of EBHSV were introduced with released hares, possibly resulting in negative impacts on indigenous populations that have not yet evolved resistance to these new virus strains (Spyrou et al. 2013). Importantly, in a study conducted in the same region (Prefectures of Chalkidiki and Thessaloniki and the island of Thasos) during 2010-2012 (Sokos et al. 2012), a widespread distribution of EBHS was revealed. This study showed that during the two years of sampling, the disease was in an endemic stability state with approximately 17% prevalence of EBHSV.

In Italy, tularemia has been detected in hares imported from Hungary and Romania (Alborali and Chiari 2010), and in Spain an outbreak of tularaemia in humans was attributed to imported hares (Pérez-Castrillón et al. 2001). In France between 1969 and 1971, the examination of 92 translocated hares from eastern Europe revealed two new records of helminths in the country: *Andrya rhopalocephala* and *Misipsella numidica* (Graber and Lebrette 1971). In southern France, the introduction of Iberian hares (*Lepus granatensis*) caused the infection of indigenous brown hares by a new parasite, the *Nematodiroides zembrae* (Bordes et al. 2007).

Stress caused during animal capture and transportation can reduce the response of the immune system and hence increase the chance of developing a certain disease following infection by pathogens (Paci et al. 2006; Dickens et al. 2010). Consequences for the wild rabbit populations have been proved in Spain. In areas where rabbits had been realeased, the indigenous animals exhibited higher pathogen infections and releases were proven to be the route of pathogen introduction into the wild populations (Navarro-Gonzalez et al. 2008).

7.6 Hare release as a management measure

Whether release is an effective management measure depends primarily on the pursued aim. Hares are released into the wild for the following reasons: a) to conserve_threatened populations and establish new populations (introduction, reintroduction) (Fischer 2011), and b) to augment hunted populations with releases after the hunting season (Pielowski 1976; Dematteis et al. 2003; Santilli and Galardi 2006).

Hare species have high reproductive rates, so local populations can recover quickly after a temporal decrease, and vacant areas can be re-colonized by dispersers from neighboring areas if the habitat is suitable (Chapman and Flux 1990; Ray 2001; Krauset al. 2005). Natal dispersal in brown hares can exceed five kilometers (Bray et al. 2007; Avril et al. 2011). However, releases for introduction or re-introduction onto islands and in other isolated areas may be necessary (Chapman and Flux 1990).

Conserving threatened hare populations with releases does not seem an appropriate management measure, and in general it has not been proposed (Smith et al. 2004; Farías et al. 2008; Rico et al. 2008). From a genetic point of view, the lack of a relationship between heterozygosity and population density of brown hare has been found in Poland (Hartl et al. 1992) and Hellas (Suchentrunk et al. 2003), indicating that homozygosity is not a limiting factor for small hare populations.

Providing game for shooting by releases just before and during the hunting season (a practice often known as 'put and take' with galliforms) is not known to be applied for hares and is not proposed (Tomasevic 1977). Released hares cost from 60 to 180 € per hare (Krapinec et al. 1999; Tume 2000), in contrast to galliforms which cost about 5 to 10 € per bird (Sokos et al. 2008). Moreover, hare hunting is a traditional activity in which hares are chased by dogs, and in many cases a run can last for 30 minutes to two hours, and this duration determines the quality of the hunt. Released hares, especially cage-reared, are not expected to display proper escape behavior (Lemnell and Lindlof 1982; Pépin and Cargnelutti 1987; Meriggi et al. 2001) for this traditional hunting method until a few days or weeks after release. Moreover, 'put and take' shooting could be characterized as an alienated and unnatural form of hunting and it raises ethical questions (Sokos et al. 2014).

Augmenting hunted populations with releases after the hunting season is the most common purpose of hare release. However, this practice indicates weaknesses in the application of sustainable hunting and other management measures to increase wild populations of species (Marboutin et al. 2003; Sokos et al. 2003). Thus for many a solution is releases of adults in late winter and usually juveniles in spring and summer (Jezierski 1968; Marboutin et al. 1990). For reared leverets introduced in spring or summer, the recovery rate by hunters varies from 3.5 to 26% (Fiechter et al. 1988a; Lemnell and Lindlof 1982; Biadi and Benmergui 1989). One exception is the recovery rate of 45% reported by Krapinec et al. (1999). Mean recovery rates are slightly higher (13-30%) for wild brown hares translocated in winter (Biadi and Benmergui 1989; Lartiges et al. 1989; Benmergui et al. 1990). Fiechter (1988b) marked 893 translocated and imported brown hares, which were released in December and January. The percentage of recovery by hunters was 15%. According to the National Hunting and Wildlife Agency of France (ONCFS 2014) the benefit of releases at the peak of this practice, where the number of released hares reached around 200,000 individuals per year, represented 1-3% of the national number of harvested hares.

Hundreds of translocated brown hares were released in January and February in three areas of Poland with local hare population densities of 11-48 hares/km², and the ratios of released to indigenous brown hares the first days after release were 7.9-47.8% (Jezierski 1968). The rate of disappearance was greater in areas with higher local brown hare densities. However, in the following hunting season, belt counts did not show any increase in population densities in any of the three areas.

Pielowski (1976) used the method of "mass introduction" with the release of a large number of translocated brown hares in early spring. He released 500 brown hares into a 40 km² area with 5.7 indigenous hares/km². The population density increased fourfold for the next two years but subsequently decreased to the before-release density values. He then released 600 hares into a second area of 62 km² with 9.2 indigenous hares/km². In this instance the density initially doubled but after nearly six months it decreased to the before-release density values.

Jezierski (1968) concluded that augmenting hunted populations with translocations after the hunting season does not benefit hunting purposes. Gaydou and Giovo (2008, 2009) calculated costs of 652 € per translocated brown hare and 900 € per reared brown hare that remains alive for more than six months. Even if the released hares survive, they contribute little to reproduction (Angelici et al. 2000), as also noted for other lagomorphs and mammals (McDowell 1955; Letty et al. 2002; Teixeira et al. 2007).

In Italy, it is common practice to capture wild hares with nets in areas where hunting is prohibited (Zones of Restocking and Capture) and to release them into hunting areas after the end of the hunting season, usually in December and January. Santilli and Galardi (2006) investigated whether these releases increase the hunting harvest in the region of Tuscany. Each year from 2001 to 2004 about 5,000 brown hares were translocated and another 3,000 reared hares were released. However, these releases did not result in increased harvest (Santilli and Galardi 2006). In this case, habitat suitability was responsible for the differences in harvest between areas (Santilli and Galardi 2006).

7.7 Discussion and Conclusions

Released hares can be obtained in different ways and each entails different costs and different abilities to survive in the wild. Intensive rearing in cages may be more profitable for farmers as production is higher than in extensive rearing in outdoor enclosures. Survival in nature is low for intensively reared hares, and improved for extensively reared hares and wild translocated hares. However, the number of hares produced extensively is small and the capture of wild hares is not an easy task. In intensive rearing, the use of juveniles and their adaptation in large outdoor enclosures can slightly increase survival rates. Predation is the main cause of mortality and consequently predation control should be accompanied with releases, using appropriate techniques and taking into account the protection of endangered carnivores (Reynolds et al. 2010).

New techniques, such as learning anti-predator behavior and predator repellents, are expected to decrease mortality and should be tested in the future. Moreover, releasing a higher number of hares in a relatively small area might mitigate the increased mortality and ensure that some individuals remain available for reproduction, if the aim is population establishment ("mass introduction", Pielowski 1976).

In any case, the practice of releasing hares, as it has been applied until now, has low benefits in relation to cost. In areas that releases were applied, hare populations and hunting harvests did not increase significantly. Releases occurring a few days before the start of, or during the hunting season, may contribute to a higher hunting harvest. However, the behavior of these hares may not be sufficient to offer a traditional quality hunt, and for some scholars it is an action contrary to hunting ethics (Sokos et al. 2008).

From an economic point of view, the release of 30 hares costs about 3,000 €. Of these 30 hares, the most optimistic survival expectancy is five animals for about five months (Table 1). Therefore, each hare that survives for a few months and has a low reproductive output will cost at least 600 €. Thus, the final result is not cost-effective. The whole system resembles an inefficient factory production process. According to the input-output analysis and the principle of “what goes in, must come out”, the production process of a factory should use all inputs (materials, energy, etc.). When quantities of materials do not become a product for the scope of the operation, then this output is defined as a non-product output. In environmental management science, the non-product outputs are wastes. The same principle can be applied to the system of intensive or cage-rearing because the production process, according to the literature, is inefficient. For example, more than 60% of the reared hares die within thirty days after release. This is clearly a 60% non-product output which bares 60% of the production costs of the process. Thus, this system produces 60% wastes, or more, when considering alien genes and pathogens. Future research could focus on a Material Flow Cost Accounting approach for hare releases (Jasch 2009). Other management practices such as predation control, habitat improvement and harvest management, are noted by many authors as more effective at increasing hare populations (Smith et al. 2004; Reynolds et al. 2010).

Consequently, if responsible agencies wish to save money, then hare releases may be appropriate only in areas where the wild hare population has disappeared and the initial causes of this decline have been found and treated. In such cases, the most appropriate choice is translocation with the parallel implementation of appropriate management measures (IUCN/SSC 2013). One alternative is the use of hares reared in large outdoor enclosures.

Apart from the low effectiveness of hare releases, the most disturbing matter is that hare releases involve risks of long-term impacts for wild populations through genetic degradation and disease transmission. The genetic diversity and evolution of hares are subject to nature conservation legislation and strategy. The European Charter on Hunting and Biodiversity (Strasbourg 2007) refers that managers and hunters should ensure that the rearing and release of animals will not introduce alien genes and will not affect the evolutionary process.

Consequently, if responsible agencies desire to conserve the genetic diversity of the species, then before each hare release Evolutionary Significant Units (ESU), should be identified combining genetic, phenotypic and ecological data (Kasapidis et al. 2005, Rico et al. 2008). In a genetic context, a combination of appropriate genetic markers should be used (Rosenberg and Nordborg 2002; Oyler-McCance and Leberg 2005; Randi 2008) and markers of mtDNA and nuclear DNA should be applied. Useful tools are microsatellites using a suitable number of hares and loci (Fickel et al. 2005), and Single Nucleotide Polymorphisms (Melo-Ferreira et al. 2009).

Hare farm stock should originate from the indigenous fauna of each ESU and hare farms should be certified for this after each capture of wild hares. Produced hares should be released only within the ESU. Moreover, intensive rearing forces the hares to move away from their eco-ethological identity, a fact that also raises animal-welfare issues. Consequently, detailed legislation on rearing practices should be adhered to by hare farms, and this raises the question as to whether hare farms should be managed by the private sector or hunting clubs without strict scientific scrutiny.

Similarly, if responsible agencies wish to protect hare species from pathogens, then the health screening protocol should include laboratory testing of animals for diseases that have been proven to cause losses in wild populations and also threaten other species and humans (Wibbelt and Frölich 2005; Mathews et al. 2006). However, no screening program can be completely exhaustive, as it is limited by cost, laboratory equipment, expertise, and the number of available samples (Mathews et al. 2006). Consequently, the transfer of animals for long distances increases the risk of disease transmission. The responsible authority should take into account the pathogens both in the region of origin and in the destination area (Mathews et al. 2006). Thus, according to the ESU, the term 'Disease Significant Units' could be proposed, to denote geographical areas where hares encounter the same pathogens and have developed corresponding immunity. Furthermore, the combination of ESU and DSU should define the 'Permitted Transferring Units' where hares should not be transferred for rearing and release from one PTU to another.

In conclusion, the rearing and release of wildlife species should be carried out according to effective wildlife conservation legislation. To meet these requirements, the existing legislation needs to be improved in many countries by the establishment of PTUs in order to protect wild populations from genetic and evolutionary degradation, and pathogen transmission. Additionally, hare releases for hunting do not deserve public funding due to their low necessity and effectiveness compared to other management measures. In specific cases, such as the introduction or reintroduction of the species, the rearing and release of hares should be under strict scientific scrutiny and only conducted following feasibility studies.

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8. GENERAL DISCUSSION AND CONCLUSIONS

There are a lot of discussion about the conservation and increase of wildlife species populations and their appropriate management, although in case of diseases the most ecologists and managers cannot reach in detailed statements and management guidelines or even in some cases diseases are not taken into account. However, the last years many researchers have begun studying ecology of wildlife diseases trying to find their impacts and wheather some management measures can contribute to their prevention. This PhD thesis has this aim in the case of an important species for hunting economy and ecosystem functions and a virus which the last three decades causes serious mortality: the brown hare and the EBHSV.

The high diversity of ecotopes in nearby areas and the free access of hunters in wide geographical areas provided the opportunity to study the prevalence of the pathogen in a Mediterranean region of about 7,000 km². The intensive sampling conducted during the study has provided detailed data on the aspects of the prevalence and the possible factors which may influence this prevalence. Additional studies revealed special aspects of hare ecology and EBHSV that are important for understanding the disease management.

The study confirmed that EBHSV had become in an endemic stability in all studied region and was found to be 17.8%, a relatively high prevalence taking into account that PCR detects the virus that has recently infect the animal and there is around and during the stage of viraemia. EBHSV prevalence was different across areas. Similarly the hunting harvest of hares was different, with more hares be harvested in diverse landscapes of shrublands and farmlands. The relation of harvest and EBHSV prevalence is not significant; this seems to be more due to the many factors that influence harvest and hare populations and probably the different transmission due to environmental factors, susceptibility and immunity of hares (Chapters 3 and 4), and also the different virulence of the virus.

Indeed, a more detailed study with the contribution of GIS found that EBHSV prevalence is higher in areas with higher hare densities, nearer to paved roads network and in lowlands. An ecotope with these characteristics is Olive groves in Chalkidiki, where EBHSV prevalence is high. GIS was proven to be a very helpful tool and thus the influence of environmental factors on diseases can be examined more efficiently.

A sex-biased prevalence was also found with double the number of males infected with EBHSV than females, indicating a sexual dimorphism in disease exposure or susceptibility. Between sex and the above three spatial factors (population, distance from roads and altidude) was not an interaction (P>0.1). Consequently, males are more infected or be susceptible and may have higher mortality that appears to contribute to a sex ratio biased towards females. More adults were found infected than young, although this was not found to be significant. Adults had a higher spleen mass indicating past infections from EBHSV and other pathogens. Enlarged spleens were more frequently found in hares positive to EBHSV hares, and a negative relation was observed between spleen mass and body condition indicating energy consumption for immune defense investment. EBHSV prevalence was not related with hare body condition, indicating that hares with higher body condition appear to have higher probability to overcome the disease.

The severe habitat disturbance by wildfire in Kassandra Peninsula caused initially a reduction in hare abundance, although after one year and for four years hares were more abundant in burned area. Wildfire seems not to influence EBHSV prevalence between burned and unburned areas one year after the wildfire. That year of 2007 is characterized by a low EBHS prevalence of 5.7% in all Kassandra Peninsula. Few years after the wildfire, in period 2010-12, the EBHSV prevalence was higher (28.6%) and also no difference was found between burned and unburned areas.

The survey methods for population estimation and samples collection is an important task in ecology of wildlife diseases and should properly designed. Spotlight counts are frequently used for hares and other mammals in Europe, however the influence of several factors, like weather and moonlight effects, is generally overlooked. Here the method was tested in Mediterranean conditions. Only wind speed and direction had an influence on hare counts tending towards significance. Thus, the researcher should expect to see fewer juvenile hares in windy days, something that would not apply to developed hares. Yet if the wind is blowing from the hares towards vehicle the researcher is likely to count more hares. Temperature and moon did not influence spotlight counts. Precipitation also had no effect possibly due to its low variability in this study.

Finally, an integrated review about hare rearing and release revealed that this practice may contribute in transmission of many important infectious diseases, including EBHS. Moreover, hare releases have not increased significantly the wild hare population or the hunting harvest in areas where the practice has been applied. The risk of genetic and evolutionary degradation and pathogen transmission is possible in wild populations. In previous studies several factors identified that differentiate EBHSV prevalence and these could be taken into account for the prevention of disease and increase of release success. Such factors are the different EBHSV prevalence between areas and the manager should avoid translocating hares from or to areas with a high prevalence. Moreover, hare densities, distance from paved road network, altitude and sex of hares are parameters that could taken into account. For example, the temporally increase of population density after a release may cause the increase of EBHSV prevelance.

APPENDICES

Appendix 1. The questionnaire that was given to hunters.

Αριθμός λαγού ☐

1) Όνομα κυνηγού:
2) Ημερομηνία εξόδου:
3) Δημοτ. διαμέρισμα που θηρεύτηκε ο λαγός (**σημειώστε στο χάρτη τον αριθμό του λαγού**):

5) Σημειώστε που ήταν το γιατάκι του λαγού:
ΕΝΤΟΣ / ΕΚΤΟΣ Ζώνης Εκπαίδευσης Σκύλων

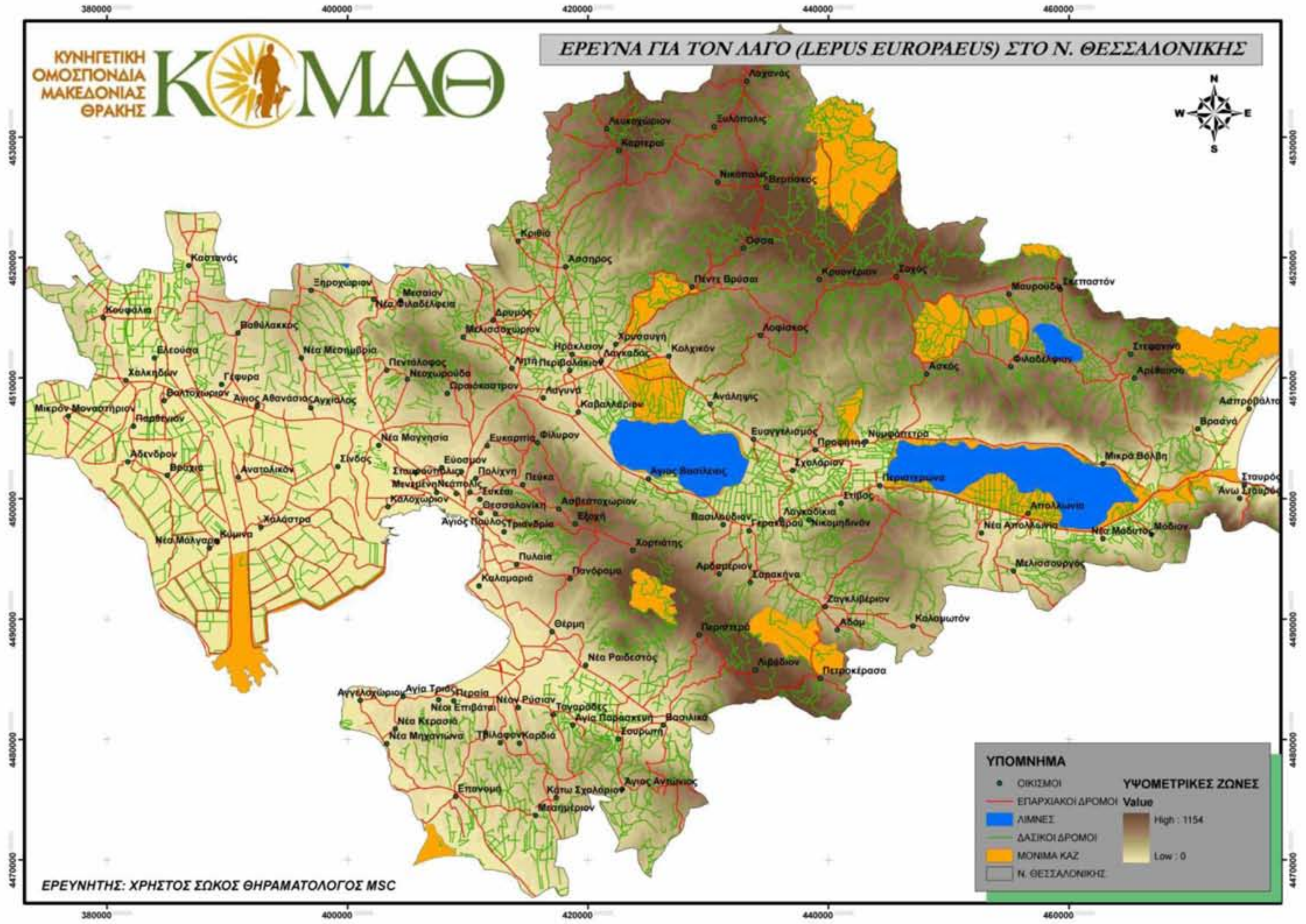
Κάτω του 1Km από καταφύγιο	1-2 Km από καταφύγιο	2-5 Km από καταφύγιο	Άνω των 5 Km από καταφύγιο

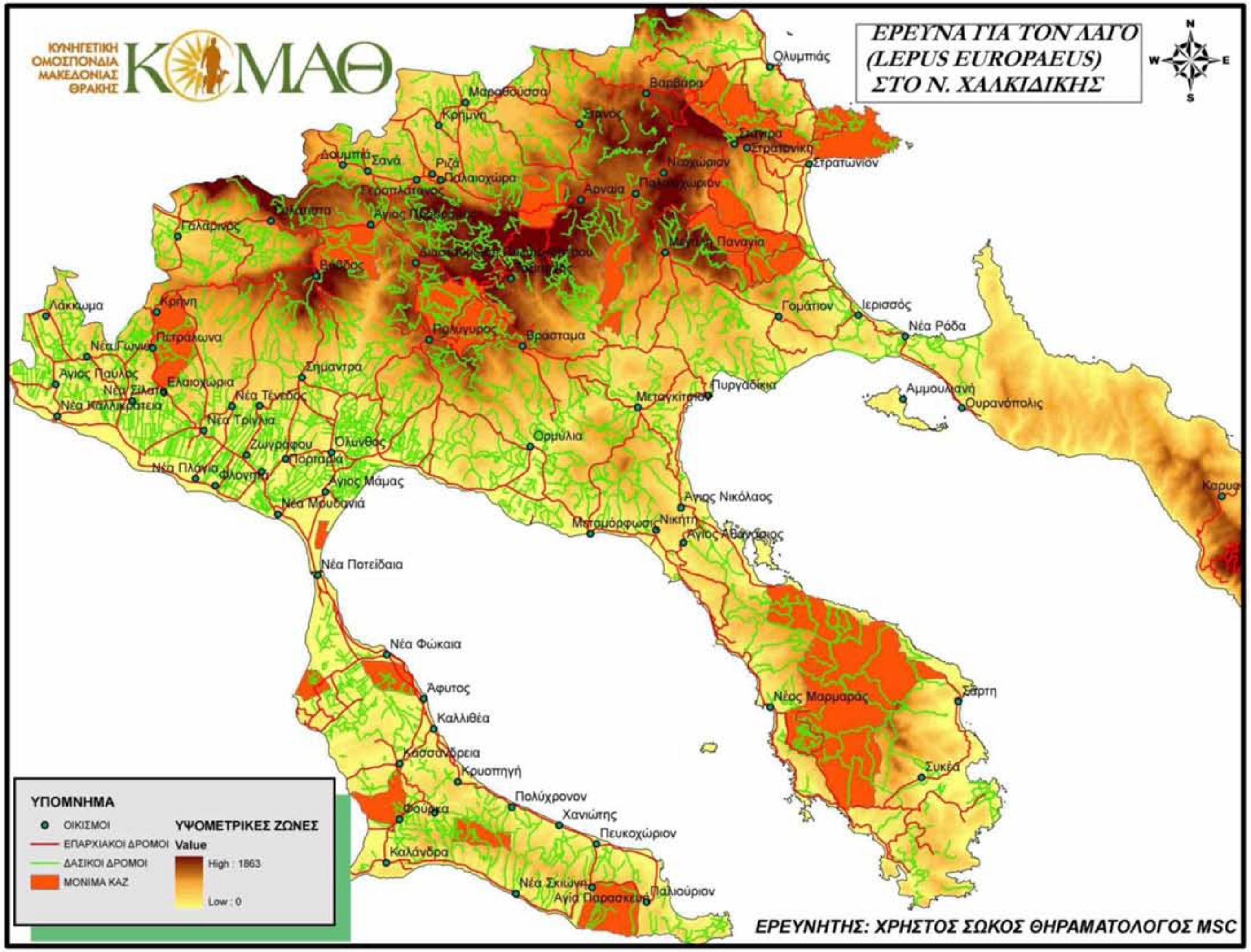
6) Σημειώστε:
- ΑΡΣΕΝΙΚΟ / ΘΗΛΥΚΟ
- Αριθμός εμβρύων (0-) ☐
- Θηλασμός

7) Η περιοχή γύρω από το γιατάκι (ακτίνα 400μέτρα) καλύπτεται κυρίως με:
- Ποτιστικές καλλιέργειες (βαμβάκι κ.α.)
- Ξηρικές καλλιέργειες (σιτηρά κ.α.)
- Θαμνότοπος (πουρναρολίβαδο)
- Δάσος (πεύκη, βελανιδιά, οξιά)

8) Συλλέγετε
Ένα **μάτι**
Κομμάτι από το συκώτι
Ένα νεφρό εάν είναι εφικτό
Τον σπλήνα εάν είναι εφικτό

Appendix 2. The maps that were given to hunters, for the indication of the site where the collected hare was firstly found.




ΚΥΝΗΓΕΤΙΚΗ ΟΜΟΣΠΟΝΔΙΑ ΜΑΚΕΔΟΝΙΑΣ ΘΡΑΚΗΣ
ΚΟΜΑΘ
ΕΡΕΥΝΑ ΓΙΑ ΤΟΝ ΛΑΓΟ (LEPUS EUROPAEUS) ΣΤΟ Ν. ΧΑΛΚΙΔΙΚΗΣ
ΥΠΟΜΝΗΜΑ
ΟΙΚΙΣΜΟΙ
ΕΠΑΡΧΙΑΚΟΙ ΔΡΟΜΟΙ
ΔΑΣΙΚΟΙ ΔΡΟΜΟΙ
ΜΟΝΙΜΑ ΚΑΖ
ΥΨΟΜΕΤΡΙΚΕΣ ΖΩΝΕΣ
Value
High : 1863
Low : 0
ΕΡΕΥΝΗΤΗΣ: ΧΡΗΣΤΟΣ ΣΩΚΟΣ ΘΗΡΑΜΑΤΟΛΟΓΟΣ MSC