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Endoparasites of the European hare (*Lepus europaeus*) (Pallas, 1778) in central ItalyV. SERGI¹, G. ROMEO², M. SERAFINI³, E. TORRETTA³, F. MACCHIONI^{1*}

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Summary

Brown hare (*Lepus europaeus*) populations in Europe have declined through decades due to several, but not clear yet, factors. Parasite infections and diseases are some of the causes that directly affected the survival and breeding rates of animal population.

A study on the endoparasites of 70 hares (37 hunted free-living hares, and 33 bred on farms hares) was performed between 2015 - 2017 in the province of Grosseto (central Italy), an area where the impact of parasites in the hare population has never been investigated. During necroscopic analysis of hunted hares the following helminthes were found: *Trichostrongylus retortaeformis* (87.1 %), *Passalurus ambiguus* (12.9 %) and *Andrya* spp. (6.4 %) in the intestinal tract, *Protostrongylus cuniculorum* (8.3 %) in lungs and *Dicrocoelium dendriticum* (16.7 %) in livers. The prevalences of the intestinal helminthes in bred hares were: 12.1 % for *Passalurus ambiguus* and 3 % for *Trichostrongylus retortaeformis*. The coprological analysis showed prevalences of 64.9 % for coccidia in the 37 hunted hares and 45.5 % in the 33 bred hares. The relationship between the intensities of parasitic infections and body weight was evaluated.

The results of the present study in the Grosseto area indicate that free-living hares have few species of parasites and that the intensities of parasitic infection did not affect their general condition and health, suggesting that endoparasites played no detectable role in the dynamics of this hare population.

Keywords: Brown hare; necroscopical analysis; coprological analysis; host-parasite

Introduction

The European hare (*Lepus europaeus*) is the most important game species in Europe and the most widespread hare species in Italy. Since the 1960s, there has been a general decrease in the number of hares in many European countries (Smith *et al.*, 2005; Marboutin *et al.*, 2003). There are several probable causes of this population decline: a reduction in the number of suitable habitats for hares due to the intensification of agriculture (Smith *et al.*, 2005), over-hunting (Meriggi *et al.*, 2001), the increase in the number of

predators, especially the red fox (*Vulpes vulpes*) (Goszczynski & Waseilewski, 1992; Knauer *et al.*, 2010), and the spread of infectious diseases such as EBHS (European brown hare syndrome), pasteurellosis, yersiniosis and tularemia (Lamarque *et al.*, 1996; Posauts *et al.*, 2015). Parasite infections can also be significant negative controlling factors of hare populations in terms of being the direct cause of death and, above all, as debilitating factors (Posauts *et al.*, 2015; Diakou *et al.*, 2014; Kornaš *et al.*, 2014; Chroust *et al.*, 2012; Dubinsky *et al.*, 2010).

The aim of the present study was to investigate the endopara-

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sites of both free-living and bred European hares in the province of Grosseto (Tuscany, central Italy), an area where the hare population has already been well studied (e.g. Santilli *et al.*, 2014; Santilli & Ferretti 2008), with the exception of the impact of parasites, which has never been investigated. In Tuscany there has been a decline of the species since the late 1960s (Santilli & Galardi 2006). The last published data revealed a density of 9 hares/km² estimated in 23 protected areas in the province of Grosseto (Santilli & Ferretti, 2008).

Materials and Methods

A total of 70 European hares were collected from 2015 to 2017 in the province of Grosseto (central Italy). 37 hares (27 females and 10 males; 7 young hares and 30 adults) were legally culled, according to Italian Law No. 157/92 and 33 hares were collected from two farms (20 females and 13 males; 7 young hares and 26 adults). All samples were taken to the Office for Hunting and Fishing in Grosseto.

The viscera were separated from the rest of the carcass by hunters or breeders, therefore in some cases not all organs of hunted hares were properly conferred. Conversely samples of all organs of bred hares were accurately collected and analysed. The samples were transferred to the Parasitology Section of the Department of Veterinary Sciences at the University of Pisa.

Individual data on the area of origin, gender, weight, and age of the hares were recorded. Hares were classified as young (≤ 8 months of age) if the Stroh's tubercle was present, otherwise they were classified as adult (>8 months of age) (Stroh, 1931).

The gastro-intestinal tracts of 31 hunted hares and 33 bred hares were analysed. Intestinal mucosa was observed macroscopically, gastric contents were submitted to sedimentation and examined under a stereomicroscope. Intestines were examined with the sedimentation and counting technique (SCT) in accordance with Eckert *et al.* (2001).

Kidneys, urinary bladders, and spleens of 31 hunted hares and 33 bred hares were opened, washed and the sediment was examined by stereomicroscopy.

The livers of 24 hunted hares and 33 bred hares were analysed by stereomicroscopy.

The cardiorespiratory system of 24 hunted hares and 33 bred hares was examined. The trachea was examined under a stereomicroscope, pulmonary tissue smears were taken to search for eggs and larvae. Heart and lungs were cut and washed with tap water in order to collect adult parasites.

All adult parasites found were isolated, counted, separated by gender and stored in 70 % alcohol and classified, according to taxonomic keys and some articles (Yamaguti 1959; Levine 1968; Soulsby 1968; Boev 1975; Durette-Desset, 1977; Audebert *et al.*, 2000).

Rectal faecal samples (at least 3g) of all 70 hares were subjected to coprological analysis to detect parasite oocysts, eggs and lar-

vae, using flotation in centrifuge with 50 % ZnCl₂ (s.g. 1.300) and K₂HgI₄ (p.s.1450) as flotation solutions, according to the procedure described by Dryden *et al.* (2005).

Coprocultures of fresh faeces of bred hares were performed to obtain the coccidian oocysts sporulation in order to allow the identification of the coccidia species. Faeces with oocysts were treated with K₂Cr₂O₇ to enable oocysts to sporulate (Eckert *et al.*, 1995). Prevalences with 95 % confidence intervals (CI), mean abundance, mean intensity, and range were calculated (Bush *et al.*, 1997). Multiple parasite infections were also described. Pearson's Chi squared test and Fisher's exact test were carried out to compare parasite prevalences in hares of different age groups, genders and provenance (i.e. free-living vs. bred hares). For the most prevalent parasite, a negative binomial distribution was compared to obtained data by Pearson's Chi square test in order to investigate the stability of host-parasites relationship as an indication of overdispersion (Bliss & Fisher, 1953). The relationship between the intensities of parasitic infections as predictor variables and body weight as a dependent variable was evaluated using Pearson correlation and the results were expressed as correlation coefficients and regression equations.

The results of the coprological tests were compared to necropsy results taken as a gold standard. The significance of the tests was confirmed for P values lower than 0.05. All the statistical analyses were carried out using Quantitative Parasitology 3.0 software (Rózsa *et al.*, 2000) and SPSS package version 20.

Ethical Approval and/or Informed Consent

The present work did not involve the use of laboratory animals. Samples were gathered from dead free-ranging brown hares which were legally shot by hunters in accordance with Italian Law (157 of 11/02/1992). Thus, no animals were killed specifically for this study.

Results

Hunted hares

The most prevalent parasites in the intestines of hunted hares were *Trichostrongylus retortaeformis* (87.1 %) in the small intestine, followed by *Passalurus ambiguus* (12.9 %) in the large intestine, and Cestoda of the family Anoplocephalidae (9.6 %) in the small intestine. On the basis of morphologic and morphometric analyses of scolex and proglottids, the cestodes were identified as *Andrya* spp. (Table 1).

Of the 31 examined hares, 12.9 % were negative to parasites, 67.7 % were positive to one parasite species, and 19.4 % presented double infections. All single infections were by *T. retortaeformis*, double infections were by *T. retortaeformis* and *P. ambiguus* (12.9 %), and by *T. retortaeformis* and *Andrya* spp. (6.5 %).

Comparing prevalences of intestinal parasites between genders, no significant differences were found. Between age classes, a sig-

Table 1. Necropsy of free-living hares *Lepus europaeus*. Intestinal and liver helminths. NP = number of positive hares, P% = Prevalence, CI %=95% confidence interval of prevalence, TP = total number of parasites, R = Range, A = Abundance; MI = Mean Intensity.

Necropsy	NP	P (%)	CI(%)	R	TP	A	MI
Hunted hares (n=37)							
Intestinal parasites^a							
<i>Trichostrongylus retortaeformis</i>	27	87.1	70.1 – 96.3	2 – 3650	18505	596.9	685.4
<i>Passalurus ambiguus</i>	4	12.9	3.6 – 29.8	23 – 664	793	25.6	198.2
<i>Andrya</i> spp.	2	6.4	0.8 – 21.4	1 – 2	3	0.1	1.5
Liver parasites^b							
<i>Dicrocoelium dendriticum</i>	4	16.7	0.8 – 21.4	16 – 610	644	26.8	161.0
Bred hares (n=33)							
Intestinal parasites							
<i>Trichostrongylus retortaeformis</i>	1	3.0	–	–	79	2.4	79
<i>Passalurus ambiguus</i>	4	12.1	3.4 – 28.2	1 – 312	370	11.2	92.5

^aThe gastro-intestinal tracts of 31 hunted hares were analysed.

^bThe livers of 24 hunted hares were analysed.

nificant difference was observed only for *T. retortaeformis* (P value of Fisher's exact test = 0.008): adult hares (25/26 hares, 96.2 %) were more infected than young hares (2/5 hares, 40.0 %).

T. retortaeformis was found with a high abundance and high intensity (597 and 685 respectively), with a high total number of parasites isolated (18505), ranging from 2 to 3650 specimens.

For *T. retortaeformis*, a negative binomial distribution, with parameters $k = 0.44$ and $p_{\text{binom}} = 0.00074$, was found to fit the data and tested (Pearson's Chi squared P value higher than 0.05). No correlation was found between body size and the intensity of infection of *T. retortaeformis* ($r = 0.291$; $p = 0.119$).

No parasites were found in the spleen nor in the urinary system.

The most prevalent parasite in the bile ducts was *Dicrocoelium dendriticum* found with a prevalence of 16.7 %. No significant differences in the prevalences between genders and age classes were observed.

In the cardiopulmonary system, no parasites were found in the hearts and tracheas of the examined hares. In the lungs of two hares, parasites of the family Protostrongylidae were found, species *Protostrongylus cuniculorum*. The nematodes were clustered

in nodules on the lung surface. Due to the difficulty of isolating parasites from nodules, it was only possible to calculate the prevalence of the infection and not the number of parasites. In addition to adult parasites, the presence of larvae was evidenced by pulmonary smears. The prevalences were not significantly different between genders and age classes of the hosts. The species identification was established on caudal bursa according to the features proposed by Boev (1975).

Coprological analysis revealed that 30 faecal samples were positive to some parasites (Table 2).

The results of the coprological test were compared to necropsy results (considered as the gold standard) in 31 hunted hares for the most prevalent parasite *Trichostrongylus retortaeformis*. The sensitivity (s) of the coprological test was 0.33 and the specificity (s') was 1.00.

A total of 24 free living hunted hares were positive to coccidia (64.9 %). There were no significant differences in the prevalences between age classes and genders. No correlation was found between body size and the intensity of infection of coccidia ($r = -0.160$; $p = 0.179$).

Table 2. Coprological examination hares *Lepus europaeus*. NP = number of positive hares, P%=prevalence, CI%=95% confidence interval of prevalence, R= Range, Mean OPG/EPG=Mean oocysts/eggs per gramme of faeces.

Coprology	NP	P%	CI%	R	Mean OPG/EPG
Hunted hares (n=37)					
Coccidia	24	64.9	47.5 – 79.8	50 – 26600	4765.2
Gastro-intestinal Strongylids	11	29.7	15.9 – 47.0	50 – 200	59.1
<i>Dicrocoelium dendriticum</i>	4	10.8	3.7 – 25.4	100 – 600	175
Bred hares (n=33)					
Coccidia	15	45.4	28.1 – 63.6	150 – 51750	5037.8

Table 3. Epidemiological studies on the prevalences of parasites in hares in Europe.

Country	EL ^a	PL ^b	AT ^c	CZ ^d	SK ^e	ES ^f	FI ^g	FR ^h
N° of hares	84	83	225	137	74	53	24	22
<i>Trichostrongylus retortaeformis</i>	50	32.5	82.7	83.2	6.8	56.6	54.2	9
<i>Passalurus ambiguus</i>	4.8	6.0	--	--	12.2	--	--	30
<i>Protostrongylus</i> spp.	1.2	4.8	37.3	18.2	--	--	33.3	--
<i>Andrya</i> spp.	--	--	4.4	2.9	--	34	--	--
<i>Dicrocoelium dendriticum</i>	9.5	--	--	--	--	11(7/64)	--	--
Coccidia	64.3	--	80.4	79.6	92	71.7	37.5	--

^a Greece - Diakou *et al.*, 2014; ^b Poland - Kornas *et al.*, 2014; ^c Austria - Chroustet *et al.*, 2012; ^d Czech Republic - Chroustet *et al.*, 2012; ^e Spain - Dubinsky *et al.*, 2010; ^f Finland - Alzaga *et al.*, 2009; ^g Slovakia - Soveri & Valtonen, 1983; ^h France - Bordes *et al.*, 2007;

Bred hares

On farms, necropsy examination of the large intestine, revealed the presence of *P. ambiguus* in four hares (12 %) with relatively low abundances and *T. retortaeformis* in one hare (3 %). All other organs were free from parasites (Tab. 1). Coprology examination revealed that 15 hares were positive only to the coccidia genus *Eimeria* spp., sometimes also with cases of large infection, with the OPG ranging between 150 – 51750 (Tab. 2). Three species of coccidia were identified in the bred hares: *Eimeria leporis*, *E. semisculpta*, and *E. europaea*.

Free-living vs. bred hares

A comparison between free-living and bred hares showed significant differences in the prevalences of *T. retortaeformis* (P value of Fisher's exact test < 0.001), and *D. dendriticum* (P value of Fisher's exact test = 0.03). For each parasite above mentioned, free-living hares (29.7 %; 10.8 %) were more infected than bred hares (3 %, 0 %).

Discussion

The intestinal helminths found in hunted hares were the nematodes *Trichostrongylus retortaeformis* and *Passalurus ambiguus*, and the cestode *Andrya* spp.

T. retortaeformis (87 %, 27/31), the dominant parasite in the present study, is a commonly encountered species in the small intestine of the European hare in Italy and in other areas of Europe. With regard to previous studies in other parts of Italy, a prevalence of 72 % (38/53) was found in Bologna (Stancampiano *et al.*, 2016), where it was the only gastro-intestinal strongylid detected. In Genoa the prevalence was 65 % (34/52) (Poglayen *et al.*, 2002), corresponding to the lowest value in Italy. In central Italy the prevalence was 75 % (54/72) (Poli *et al.*, 1991). Canestri-Trotti *et al.* (1988) compared the prevalence of this helminth in autochthonous and imported European hares. They found a prevalence of 71.9 % (59/82) in Ferrara, 90 % (29/30) in Modena, and 100 % (25/25) in hares imported from Czechoslovakia, Poland and Hungary. In the

province of Pisa, a prevalence of *Trichostrongylus* spp. of 76.25 % (22/29), accompanied by catarrhal enteritis was reported by Agrimi *et al.* (1981).

Considering results obtained in other European countries, we found prevalences similar to those detected in Austria and Czech Republic, while elsewhere *T. retortaeformis* prevalences were lower (Table 3).

We found that the distribution of *T. retortaeformis* in the host population had a negative binomial distribution, indicating a good ecological balance and equilibrium between parasites and hosts. The distribution pattern of parasites in host population has many involvements in epidemiologic studies and host-parasite dynamics (Anderson & May, 1978; Poulin, 1993). For macro-parasites, host morbidity and mortality are strictly density-dependent and the effects will be high when parasites follow a random distribution (with a low level of variance) in host population. Consequently, an aggregate distribution (with high level of variance) positively affects the impact of parasite in host population supporting a stable interaction (Anderson & May, 1978; May & Anderson, 1978). The prevalence of the parasite was significantly higher in adults than in young hosts, in accordance with other studies (Stancampiano *et al.*, 2016).

The importance of this nematode as a pathogenic agent that induces mortality or weight loss has been demonstrated by Gottschalk (1973), Haupt & Hartung (1977) and by Boch & Schneidawind (1988). Newly *et al.* (2005) reported that *T. retortaeformis* infection involves a strong reduction in the physical condition and fertility of females, but does not affect their survival. However, our results suggest that the intensity of parasitic infection not affect hare body size.

P. ambiguus was found in 12.9 % of hares (4/31), higher than in other studies performed in Italy. In Genoa, the percentage was 3.8 % (2/52) (Poglayen *et al.*, 2002). Percentages of 7.3 % (6/82) and 10.0 % (3/30) were found in the provinces of Ferrara and Modena respectively, while the parasite was not found in 25 imported hares (Canestri-Trotti *et al.*, 1988).

The nematode, specific to lagomorphs, lives in the cecum and anterior colon and usually does not show clinically evident signs

(Diakou *et al.*, 2014). It is rarely found in European hares and always with low prevalences, while it is more commonly found in wild rabbits (*Oryctolagus cuniculus*).

In Europe *P. ambiguous* had higher prevalences only in France (Table 3).

Andrya spp. was found in this study, with a low prevalence 6.4 % (2/31). Previous studies in Italy showed the presence of the *Andrya* genus in Ravenna (Zanni *et al.*, 1995). *A. rhopalocephala* and *Cittotaenia pectinata* were found in Pisa (Poli *et al.*, 1988). *A. rhopalocephala* was found in 3/30 hares (10 %) in Modena and in 1/25 imported hares (4 %) (Canestri-Trotti *et al.*, 1988). *Andrya cunicoli* was found in 9/137 imported hares (6.5 %) in Ferrara and Modena (Canestri-Trotti *et al.*, 1988).

In Europe *Andrya* spp. had higher prevalences only in Spain (Table 3).

Anoplocephalidae infestation generally does not cause any symptoms, only in case of massive infestations digestive disorders can occur (Heintzelmann-Grongroft, 1976).

In the present study *P. cuniculorum* was found in 8.3 % (2/24 hares). *P. pulmonalis* is the most common pulmonary parasite in Italy and Europe. However, in some locations in Tuscany the parasite *P. cuniculorum* (Joyeux & Gaud, 1946) was found in hares with pulmonary protostrongylosis (Barbiera, 1960).

P. rufescens and *P. pulmonalis* were found in some repopulation and hunting areas in Pisa (Poli *et al.*, 1988). Furthermore, *Protostrongylus* spp. was found in 2.7 % (2/72) hares in the same area (Poli *et al.*, 1991). *Protostrongylus* spp. was found in *L. europaeus* with a prevalence 14 % in Ravenna (Zanni *et al.*, 1995).

In Europe *Protostrongylus* spp. prevalences are very variable (Table 3).

The presence of pulmonary strongyles often predisposes to secondary bacterial infections, leading to alterations in lung capacity and function, which considerably reduce the possibility of escape of the hare and therefore its chances of survival (Spagnesi & Trocchi, 1992).

Dicrocoelium dendriticum was found in four livers of the 24 examined (16.6 %). *Dicrocoelium* spp. is mainly widespread among ruminants in pasture, but little known and often underestimated (Otranto & Traversa, 2002). Hares may occasionally be infected in areas of ruminant grazing (Chroust *et al.*, 2012). *D. dendriticum* was found for the first time in hares in Italy in one European hare, with prevalence 1.3 % (1/72) (Poli *et al.*, 1991).

It has also rarely been found in other areas of Europe (Table 3).

Coccidia had similar prevalence among our samples: 64.9 % (24/37) in hunted hares and 45.4 % (15/32) in bred hares. Genoa has the lowest prevalence (17.3 %, 9/52) (Poglayen *et al.*, 2002). Tacconi *et al.*, (1995) reported that in Umbria all faecal specimens of bred hares were negative for coccidia (0/867), while all free living hares in protected areas were positive (n = 1233). In central Italy, a prevalence 90.3 % (65/72) was found (Poli *et al.*, 1991). Coccidiosis was also found in the province of Pisa in 26.2 % (8/29) of dead hares (Agrimi *et al.*, 1981).

In Europe coccidia prevalences are generally high (Table 3).

Coccidia are among the most dangerous pathogenic parasites in hares. The combination of intestinal nematodes and coccidia are one of the major controlling factors in harbour populations (Chroust, 1984). Especially in young animals, a high level of infection can lead to severe illness and eventually to the death of the animal. However, our results suggest that the intensity of parasitic infection not affect hare body size.

By comparing the parasitic fauna of the free hares with the bred ones, the free hares were statistically more infected by parasites. These results could be expected due to the veterinary control of bred hares and the absence of intermediate hosts for the pulmonary strongyloidiasis. Furthermore, the risk of contracting bacteria and viral infections and parasites could increase based on the type of breeding farm (e.g. extensive or intensive farming) and represent an important limiting factor (Spagnesi & Trocchi, 1992).

Conclusion

Since the 1960s, the decrease in hare populations in Europe has led to management actions aimed at halting this reduction (Marboutin *et al.*, 2003). Fluctuations in the hare population have been attributed to many factors. However, the deterioration of animal health, strongly influenced by anthropization of the landscape, seems to be the key factor. The population density may also be influenced by various diseases, as well as by parasitic infections (Alzaga *et al.*, 2009). The results of the present study in the Grosseto area indicate that free-living hares have few species of parasites and that the intensities of parasitic infection did not affect their general condition and health, suggesting that endoparasites played no detectable role in the dynamics of this hare population. The aggregation of the most abundance parasite found (i.e. *T. re-tortaeformis*) suggests that only a minimal part of host population would, if ever, be influenced by this infection.

The situation observed in our study area, and in particular low parasite diversity, is consistent with the low host density (9 hares/km²), that probably makes parasite transmission more difficult. The critical situation of host population parallels with the low biodiversity observed in parasite community. This could eventually induce a harmful loop, since biodiversity is considered a stabilizing factor in ecological webs and the lack of specific brown hare parasites in biocoenosis may be a predisposing factor for the occurrence of exogenous and potentially dangerous parasite *taxa* in the hare population (Hudson *et al.*, 2006). Parasite community has probably suffered, directly (environmental mechanisms acting on both host and parasites) or indirectly (mechanisms acting on host density and therefore on parasite transmission), the same unknown cause of brown hare decline.

Nevertheless, the good health status of the bred hares highlights a good health management of the farms. However, an in-depth analysis of the coccidia species is needed, as they had the highest prevalences in the sample studied.

Conflict of interest

Authors state no conflict of interest

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