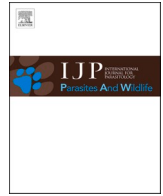




Contents lists available at ScienceDirect

International Journal for Parasitology: Parasites and Wildlife

journal homepage: [www.elsevier.com/locate/ijppaw](http://www.elsevier.com/locate/ijppaw)

# Helminth biocoenosis of *Lepus europaeus meridiei* (Hilzheimer, 1906) from Pianosa island, Italy

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## ARTICLE INFO

### Keywords:

Brown hare  
Pianosa island  
*Protostrongylus oryctolagi*  
Helminths  
*Lepus europaeus meridiei*  
Insularity

## ABSTRACT

Pianosa is a 10 km<sup>2</sup> Italian island in the Tyrrhenian Sea which is part of the Tuscan Archipelago National Park. In this island lives a brown hare population which, according to the literature, belongs to the ancestral taxon *Lepus europaeus meridiei* that offers a unique opportunity to observe how the parasite biocoenosis shapes in condition of isolation, limited space availability and high population density. The aim of this work is to describe the helminth component community of a non-managed, isolated, and dense hare population, evaluating host-parasite relationship and parasite community structure. All 26 analyzed hares (13 males and 13 females) were in good physical conditions, and all of them harboured exclusively the nematode *Protostrongylus oryctolagi* only. This is the first report of this lungworm species in Italy. The estimated overall abundance was 48.15 worms per examined hare (range 3–258, median 50) and the parasites were unevenly distributed across host population, with few hosts having most parasites (aggregated or overdispersed distribution). No significant relationship was detected between the number of isolated parasites and hare sex and weigh. The effect of the isolation of Pianosa's hare population seems to have acted reducing parasite richness, while the high host density is probably the cause of the high prevalence and abundance of the single helminth species collected.

In conclusion, despite the low impact of parasites confirmed also by the overdispersed parasite distribution, the low diversity of the studied parasite community sounds a warning for the management of the hare population and the whole Pianosa's ecosystem.

## 1. Introduction

The brown hare (*Lepus europaeus* Pallas, 1778) is a common wildlife species, representing both a target of hunting activity (Hacklander and Schai-Braun, 2019) and an important species of conservation concern in Europe, where it is classified by the IUCN as least concern with a decreasing trend (Hacklander and Schai-Braun, 2019) since the reduction of its populations in many countries (Smith et al., 2005; Pavliska et al., 2018).

The history of the genus *Lepus* in Europe is complex: natural events of dispersion, isolation, and adaptation in the late Pleistocene were followed by recent translocation of individuals mainly for restocking purposes (Canu et al., 2013). According to Mengoni et al. (2015), all these events had led to the current genetic complexity of the genus *Lepus* in

Europe (especially for the species *L. europaeus*), and probably caused the partial extinction of the subspecies *L. europaeus meridiei*, once present in northern and central Italy, northern Croatia, and south-eastern France (Amori et al., 1996, 1999; Angelici, 1998; Pierpaoli et al., 1999; Riga et al., 2001), which represents an ancestral taxon of the species *L. europaeus* (Canu et al., 2013).

In 2018, Mengoni et al. (2018) by means of a variety of genetic tools (microsatellites and mitochondrial DNA variability) identified a surviving natural reservoir of *L. europaeus meridiei* in the Pianosa island that is part of the Tuscan Archipelago National Park (Italy). Although the origin of the hare population of Pianosa is not clear, the historical reports of possible introductions date back at least to the first decade of the Twentieth century (Mengoni et al., 2018). The Pianosa brown hares, therefore, offer a unique opportunity to study how the parasite

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<https://doi.org/10.1016/j.ijppaw.2022.08.008>

Received 29 June 2022; Received in revised form 22 August 2022; Accepted 22 August 2022

Available online 2 September 2022

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biocoenosis shapes in condition of long-lasting isolation, limited space availability (1030 ha) and high population density (about 260 hares: 0.26 hare/ha).

Parasite communities, or biocoenosis (Mehlhorn, 2008), can be defined at different and nested spatial level: according to Bush et al. (1997) the first two levels are the infracommunity (the community of parasites in a single host individual) and the component community (the community of parasites in a host population or in a subset of a host species). The study of a parasite community includes the measure of its diversity, the description of its structure with dominant, codominant and satellite species and the evaluation of the possible interactions among parasite populations (Esch et al., 1990; Bush et al., 1997). Diversity describes the composition of a community both in terms of the number of species (richness) and in terms of the relative evenness of distribution of each species (Bush et al., 1997; Magurran, 2004; Poulin, 2015). Parasites are increasingly considered not only a part of the ecosystems, but also mandatory for their functioning and even a possible target for conservation (Hudson et al., 2006; Gomez and Nichols, 2013).

Parasite community structure and diversity are the result of a long and continuing interaction among host and parasite populations (Esch et al., 1990), and are affected by evolutionary, environmental, historical and stochastic factors. Therefore, which and how many parasite species are present in a host population varies for reasons that are still debated (Loker and Hofkin, 2015). Among others, host population density and isolation (narrow host geographic range) seem to act increasing and decreasing, respectively, parasite species richness (Bordes and Morand, 2011).

European hare can host several helminth species comprising nematodes, cestodes and trematodes. Among them, these of the genus *Protostrongylus* (Nematoda: Protostrongylidae) are a well-known source of pulmonary infections but are quite poorly studied from an ecological and biological point of view; it is probably due to their species-specificity, their difficult isolation and because of this genus is typical of lagomorphs and some ruminants only (Boev, 1975), and does not infect the main domestic species (cattle, horses, pigs).

According to Boev (1975), the *Protostrongylus* genus can be morphologically divided into three subgenera: *Pulmostrongylus*, *Protostrongylus* and *Davtianstrongylus*. This author reports seven species from lagomorphs: *Protostrongylus kamenskyi*, *P. pulmonalis* (sin. *P. commutatus*) and *P. boughtoni* (subgenus *Pulmostrongylus*); *Protostrongylus cuniculorum*, *P. oryctolagi*, *P. tauricus* and *P. terminalis* (subgenus *Protostrongylus*). The above-mentioned species are specific for lagomorphs and cannot be found in any other hosts. In addition, Eslami et al. (2000) isolated *Protostrongylus raillietii*, typical of ruminants, from *Lepus capensis* in Iran.

*Protostrongylus* spp. have an indirect life cycle (Anderson, 2000; Deplazes et al., 2016) with the adults living in the lung tissue. After mating, the female produces larvated eggs, that quickly hatch releasing the first larval stage (L1). L1 reaches the pharynx (both actively and helped by host coughing) and is therefore swallowed and released in the environment with the host faeces. The environmental L1 actively penetrates the intermediate host where it mutates to the second and third larval stage (L2 and L3). The spectrum of their intermediate hosts is broad and include various genera of snails or, less frequently, slugs (Lesage et al., 2015; Deplazes et al., 2016). L3 is the infective stage and usually survives in the snail until it is ingested by the final host, although some authors have reported that infective larvae can leave the intermediate host (Anderson, 2000). After the ingestion, L3 reaches the intestine and therefore migrates through the lymphatic system to the mesenteric lymph nodes, where it changes to the final larval stage L4. The L4 migrates through the blood to the respiratory system where it becomes adult in small bronchi and alveoli.

While the diagnosis of Protostrongylidae infection is quite easy, the morphological identification of *Protostrongylus* species, essential for the study of parasite epidemiology and community ecology, is quite complex and should be better supported by molecular analysis. In particular,

because of its structure and polymorphism, the internal transcribed spacer (ITS) of nuclear ribosomal DNA has become one of the most sequenced regions to identify a variety of organisms at species level, and it was already successfully used for species identification of different nematodes belonging to *Protostrongylus* genus: *P. rufescens* (Jabbar et al., 2013); *P. oryctolagi* and *P. pulmonalis* (Lesage et al., 2014).

The aim of this work is to describe the helminth component community of a non-managed, isolated, and dense hare population, evaluating host-parasite relationship and parasite community structure.

## 2. Materials & methods

### 2.1. Study area

The island of Pianosa (Livorno, Tuscany, Long. 10° 04' 44" E; Lat. 42° 35' 07" N) is part of the Tuscan Archipelago National Park (Parco Nazionale dell'Arcipelago Toscano) (<https://www.islepark.it>). From 1856 to 1998 the island was the site of a State Prison, which was subsequently decommissioned. The island is characterized by Mediterranean scrubland habitat and hosts a small population of European brown hare (*L. europaeus meridiei*), estimated at about 260 individuals in the sampling period (26 hares/Km<sup>2</sup>). In this period the Pianosa hare population was not yet recognized as the subspecies *L. europaeus meridiei*.

### 2.2. Parasitological analyses

Hares here analyzed were legally shot in Pianosa island from June 03, 2016 to October 03, 2016 as a part of the intervention planned in the initial steps of the LIFE13 NAT/IT/000471 project - RESTO CON LIFE "Island conservation in Tuscany, restoring habitat not only for birds" (<https://www.restoconlife.eu/it/the-project/>). Aim of this project was to restore the natural island communities by means of a series of measures including the eradication of non-indigenous species like the brown hare *Lepus europaeus*.

The lungs of twenty-six hares (13 males and 13 females), and the and gastrointestinal tracts of eight hares were collected and stored at -20 °C. According to Usai et al. (2012), the gastrointestinal tract was longitudinally opened, its mucosa gently scraped with a microscope slide to allow the detaching of parasites and washed with tap water. The content was than collected in conical flasks and repeatedly washed in order to obtain the sediment to be screened under a stereomicroscope for parasite collection.

Lungs were macroscopically examined, the trachea and bronchi opened with a scissor and the whole organs squeezed and washed in tap water. The sediment was examined under a stereomicroscope to observe nematode larvae. For the collection of adult parasites, according to Lesage et al. (2014) the lungs were therefore teared in small pieces (1–2 cm) and vigorously shaken in a tap water-filled jar with screw cap, whose content was then collected in conical flasks to obtain the sediment to be screened under a stereomicroscope for adult parasite collection. Collected adult helminths were fixed in 70% ethanol. All isolated nematodes were classified as male or female and counted. The caudal portion of each male was clarified in lactophenol and morphologically identified according to the key and descriptions of Boev (1975) and the descriptions of Casanova et al. (1999), Lesage et al. (2014) and Panayotova-Pencheva et al. (2018).

### 2.3. Statistical analyses

Statistical analyses were performed with Stata 12.0. Generalized linear models (negative binomial regression) were built having male parasites as dependent variable and host sex and full weight as covariates. A model with constant term only was also fitted in order to evaluate if the distribution actually differed from a Poisson and to estimate the k parameter of the negative binomial distribution (inversely related to parasite aggregation). The maximum prevalence of

undetected parasites was calculated according to Cannon and Roe (1982) considering a finite population of 250 hares.

#### 2.4. Molecular analyses

The middle or anterior part of 20 male worms from four different hares was stored at  $-20^{\circ}\text{C}$  for molecular analysis. DNA was extracted by Macherey-Nagel NucleoSpin™ gDNA Clean-up kit according to the manufacturer's instruction. The PCR of whole ITS region was performed with 50 ng of gDNA, 10 pmol each of the NC5/NC2 primer couple (Hung et al., 1999), 12,5  $\mu\text{l}$  of DreamTaq Green PCR Master Mix (Thermo Scientific) and PCR-grade water up to 25  $\mu\text{l}$  of final reaction volume. The thermal profile consisted in an initial denaturation step at  $94^{\circ}\text{C}$  for 5', followed by 30 cycles of  $94^{\circ}\text{C}$  for 30",  $50^{\circ}\text{C}$  for 30",  $72^{\circ}\text{C}$  for 90", and a final prolonged elongation step at  $72^{\circ}\text{C}$  for 5'. PCR products were then sequenced in both direction with the same primers used for the amplification at the StarSEQ facility (Germany).

The obtained sequences were edited and aligned by MEGA11 (Tamura et al., 2021), and then compared with those available in GenBank database (<https://ncbi.nlm.nih.gov/genbank>).

### 3. Results

The average weight of hares was 3464.23g (standard deviation: 328.62g) and ranged from 2500g to 4030g. Twelve out of 13 females were pregnant. All the collected hares harboured protostrongyles, as demonstrated by both typical lung lesions and the presence of parasite larvae or larvated eggs at the microscopical observation of fluid collected from the lung (Fig. 1). The isolation of adult parasites was performed from 20 out of 26 hares. The gastrointestinal tract has been collected and therefore examined in eight hares only and none of them had gastrointestinal helminths.

The main descriptive statistics about parasitological results are reported in Table 1.

All the adult males were morphologically identified as *Protostrongylus oryctolagi*. The morphological identification (Fig. 2) was confirmed by molecular analysis: sequences obtained brought to light a unique conserved sequence of 1113bp (GenBank accession number OM307447), which showed 100% identity with *P. oryctolagi* (reference sequence KJ450993).

The distribution of male parasites in host population was aggregated and fitted a negative binomial distribution with parameter k equal to 0.71 (95% confidence interval: 0.41–1.23).

Parasite abundance had no significant relationship with hare sex and weight, as demonstrated through the negative binomial regression analysis (Table 2).

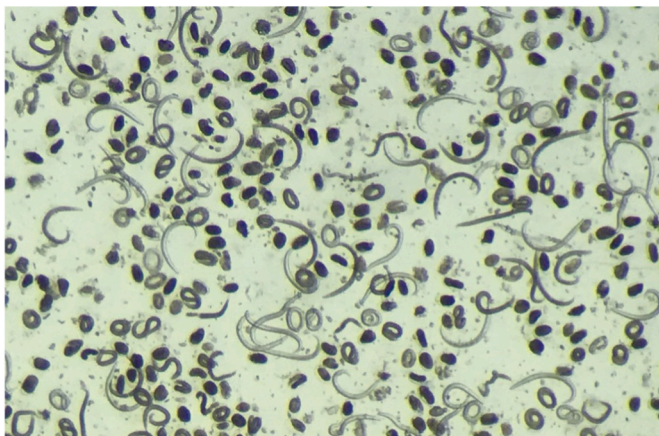


Fig. 1. Larvae and larvated eggs of *Protostrongylus oryctolagi* in lung fluid.

Table 1

Descriptive statistics of main parasitological results.

	Obs	Prevalence % (Maximum prevalence %)	Abundance (sd)	Min- Max	Median
Protostrongilidae larvae	26	100	–		
<i>P. oryctolagi</i> females	20	70	11.60 (18.30)	0–76	3
<i>P. oryctolagi</i> males	20	100	36.55 (44.99)	1–182	21.5
<i>P. oryctolagi</i> total	20	100	48.15 (62.06)	3–258	50
Intestinal parasites	8	0 (31)	0		

Obs = number of examined hare; sd = standard deviation; Min-Max: minimum and maximum number of parasites per hosts.



Fig. 2. *Protostrongylus oryctolagi*: tail of adult male.

Table 2

Negative binomial regression model performed on *Protostrongylus oryctolagi* male abundance as dependent variable.

<i>P. oryctolagi</i>	Coefficient	p-value	95% CI	
Sex	0.2868	0.679	–1.0734	1.6470
Weight	–0.0001	0.911	–0.0020	0.0018
Constant	3.7896	0.300	–0.2219	0.8890

CI = confidence interval.

### 4. Discussion

This is the first report of *Protostrongylus oryctolagi* in brown hare in Italy. The morphological identification is fully supported by the sequencing approach, confirming the ability of ITS region to discriminate among nematodes belonging to *Protostrongylus* genus, as previously reported in several studies (Jabbar et al., 2013; Lesage et al., 2014).

Observing the geographical distribution of brown hare's *Protostrongylus* species recorded in Europe, *P. pulmonalis* seems to show a preference for the northernmost regions, being reported in Finland (Soveri and Valtonen, 1983), Poland (Kornas et al., 2014), in the Czech Republic and in Austria (Chroust et al., 2012), in France (Lesage et al., 2014) and Northeast Italy (Costantini et al., 1990).

On the contrary, *P. tauricus* and *P. cuniculorum* seem to be distributed in southern regions: *P. tauricus* was reported in Spain (Casanova et al.,

1999) and in Bulgaria (Panayotova-Pencheva et al., 2014, 2018) while *P. cuniculorum* was found in Italy (Sergi et al., 2018) and in Bulgaria (Panayotova-Pencheva et al., 2018). The presence of *P. oryctolagi* in Italy, together with the only other available record of this species from *L. europaeus* in the south of France (Lesage et al., 2014) suggests a distribution in southernmost regions also for this species. The observed geographical distribution of *Protostrongylus* spp. if confirmed by further studies, could be the result of a process of adaptation to local environment, including available intermediate hosts (slugs and snails). This hypothesis suggests that the species-specificity of *Protostrongylus* spp. to their intermediate hosts could be stronger than expected (Lesage et al., 2015), and should be verified deepening the knowledge of the intermediate host-parasite relationships and their related geographical distribution.

The viability of the hare population living in Pianosa during the sampling period was confirmed by the good weight of the animals and by pregnancy of most females. Notwithstanding this viability and the high population density, the parasite community appears depauperate and dominated by a single nematode species: *P. oryctolagi*. Its 100% prevalence can be explained by the high density of the host population coupled with intermediate hosts availability and the presence of suitable habitats. Snails belonging to *Ceruellia* spp. have been identified as suitable intermediate hosts in France (Lesage et al., 2014), and *Ceruellia virgata* has been reported in Pianosa (Manganelli et al., 2014). However, further studies are needed to assess *P. oryctolagi* life cycle in the complex and sensitive Pianosa's ecosystem, with special attention to the identification of its intermediate hosts.

As regards abundance, in our best knowledge, no authors had published any survey assessing the number of adult *Protostrongylus* spp. in definitive hosts. Few papers about parasite biocoenosis in *Lepus europaeus* from Italy are available, but they all report a richer biocoenosis (see Sergi et al., 2018 for a recent study and revision of Italian literature). The presence of a single helminth species in the brown hare population from Pianosa is consistent with its geographic isolation, being the sea a barrier to migration, and it is probably the consequence of the introduction of a little number of subjects accidentally harbouring *P. oryctolagi* only (Esch et al., 1990; Bordes and Morand, 2011; Loker and Hofkin, 2015). The well-known aggregate parasite distribution within host population implies, in fact, a high probability for individual hosts to have few or no parasites of a certain species. However, the absence of trematodes, cestodes and above the intestinal nematode *Trichostrongylus retortaeformis* is quite intriguing: *T. retortaeformis* appears to be widespread, highly prevalent (from 65 to 100%) and abundant in *Lepus europaeus* Italian populations (Sergi et al., 2018), probably thanks to its adaptation to the host and to the direct life cycle that allows its persistence in absence of specific intermediate hosts. On the contrary, our survey indicates its possible absence and a 31% estimated maximum prevalence in Pianosa (Table 1). It is possible that Pianosa dry climate and the absence of wet pasture did not allow infective free-living larvae of *T. retortaeformis* to survive, whereas *P. oryctolagi* larvae, protected by intermediate hosts, were able to complete their cycle and to persist. The effect of the isolation of Pianosa's hare population, therefore, seems to have acted reducing parasite richness, despite the high host density, being the latter probably the cause of the high prevalence and abundance of the single helminth species collected (Goüy de Bellocq et al., 2002).

According to the latest studies, the interactions among parasites within a community are more frequent than expected (Ferrari et al., 2008; Stancampiano et al., 2010; Fenton et al., 2014). These interactions can help stabilizing parasite communities and hosts, making both less susceptible to alien parasite invasions (Romeo et al., 2013) and to perturbations such as natural or induced demographic fluctuations (Knowles et al., 2013). Indeed, there is growing evidence that parasite richness is related to healthy ecosystems (Hudson et al., 2006; Johnson et al., 2013).

Despite the healthy status of the host population, and the low impact

of parasites confirmed also by the overdispersed parasite distribution that acts stabilizing host-parasite relationship (Anderson and May 1978), the low diversity of the studied parasite community sounds a warning for the management of the hare population and the whole Pianosa's ecosystem.

## Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

## Declaration of competing interest

None.

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