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Protostrongylids nematodes as a tool to study the biogeography of wild mammals

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Abstract

The European brown hare (*Lepus europaeus* Pallas, 1778) is an important game species, distributed across Europe and introduced in other regions (i.e. North and South America, Australia, New Zealand). Recently, a geographically isolated population, closely related to an ancestral lineage of *Lepus europaeus meridiei*, was found on Pianosa Island, off the coast of Tuscany, Italy (Mengoni *et al*., 2018). Thus, the unique opportunity to explore the evolution and genetic structure of its helminth parasites was added to its exceptional isolation condition.

Various lungworm species within the genus *Protostrongylus* (Nematoda: Protostrongylidae) are described in European brown hares.

Our aim was to analyze the parasite population through morphological and molecular approaches in order to study the biogeography of the European brown hares (*L. e. meridiei*) population from Pianosa Island. Moreover, we investigated the morphology of a monospecific genus, i.e. *Orthostrongylus*, considering its quite intrigant descriptive history and its still unclear and debated classification.

Nuclear and mitochondrial markers were used based on their resolution power and expected polymorphism; the whole Internal Transcribed Spacer 1 and 2 (ITS), including the 5.8S rRNA sequence and the Large Subunit (28S) were used, as nuclear genes, for confirmation of the species identification. Conversely, the cytochrome oxidase c subunit I (COI) was used, as mithocondrial genes, to assess interspecific genetic relationships.

Molecular analysis corroborated the morphological identification since all the generated ITS and LSU sequences were 100% consistent with the species *Protostrongylus oryctolagi* and *Orthostrongylus macrotis*.

The paucity of molecular data existent about this genus of parasites underlines the need for more insight's studies. An in-depth analysis of broncho-pulmonary parasites and the host-parasites relationships along with the improvement of the use of mitochondrial genes, as well as the assessment of new polymorphic markers could contribute to an extensive understanding of parasites fauna and taxonomy, as well as their relationship with wild mammals' hosts.

CHAPTER I

Introduction

1.1 History and Taxonomy of Lagomorpha

"Lagomorphs" is the colloquial term used to describe mammals belonging to the Lagomorpha order. They represent a deeply defined lineage and the term itself identifies their morphology: lagos (λαγος), "hare", and morphē (μορφή), "form".

Lagomorphs are a distinct lineage of mammals whose definition “lagomorph” is a circular reference meaning “hare-shaped”. Ancestral lagomorphs evolved following the Cretaceous-Paleogene (K-Pg) boundary 53 million years ago and are in the same major mammalian clade as rodents and primates (Euarchontoglires) (O’Leary *et al*., 2013).

The order comprises 91 living species divided into two families (Figure 1) (Hoffmann and Smith 2005; Alves and Hacklander 2008): 1) the pikas (family Ochotonidae) and 2) the rabbits, hares and jackrabbits (family Leporidae). However, the systematics of the order is unclear and currently under review by the IUCN Species Survival Commission (SSC) Global Mammal Assessment and Lagomorph Specialist Group (LSG).

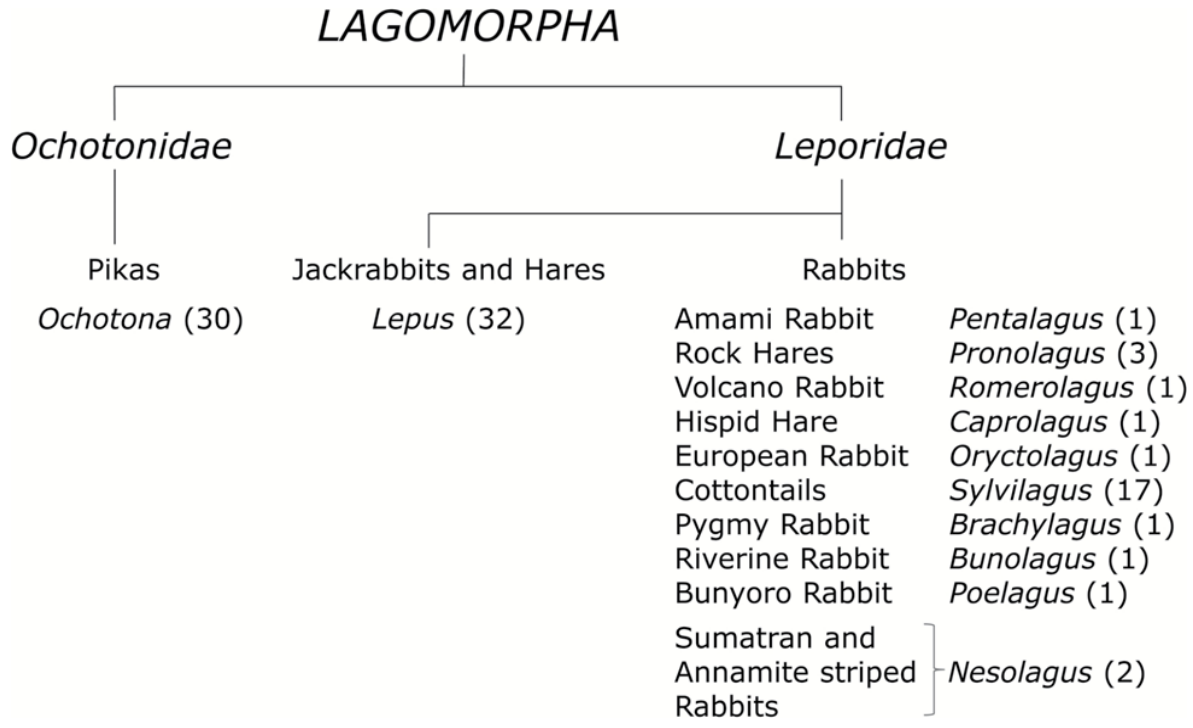


Fig. 1 – Overview of systematics of the order Lagomorpha (number in parenthesis indicates the number of species within the genera; Hoffmann and Smith 2005; Alves and Hacklander 2008).

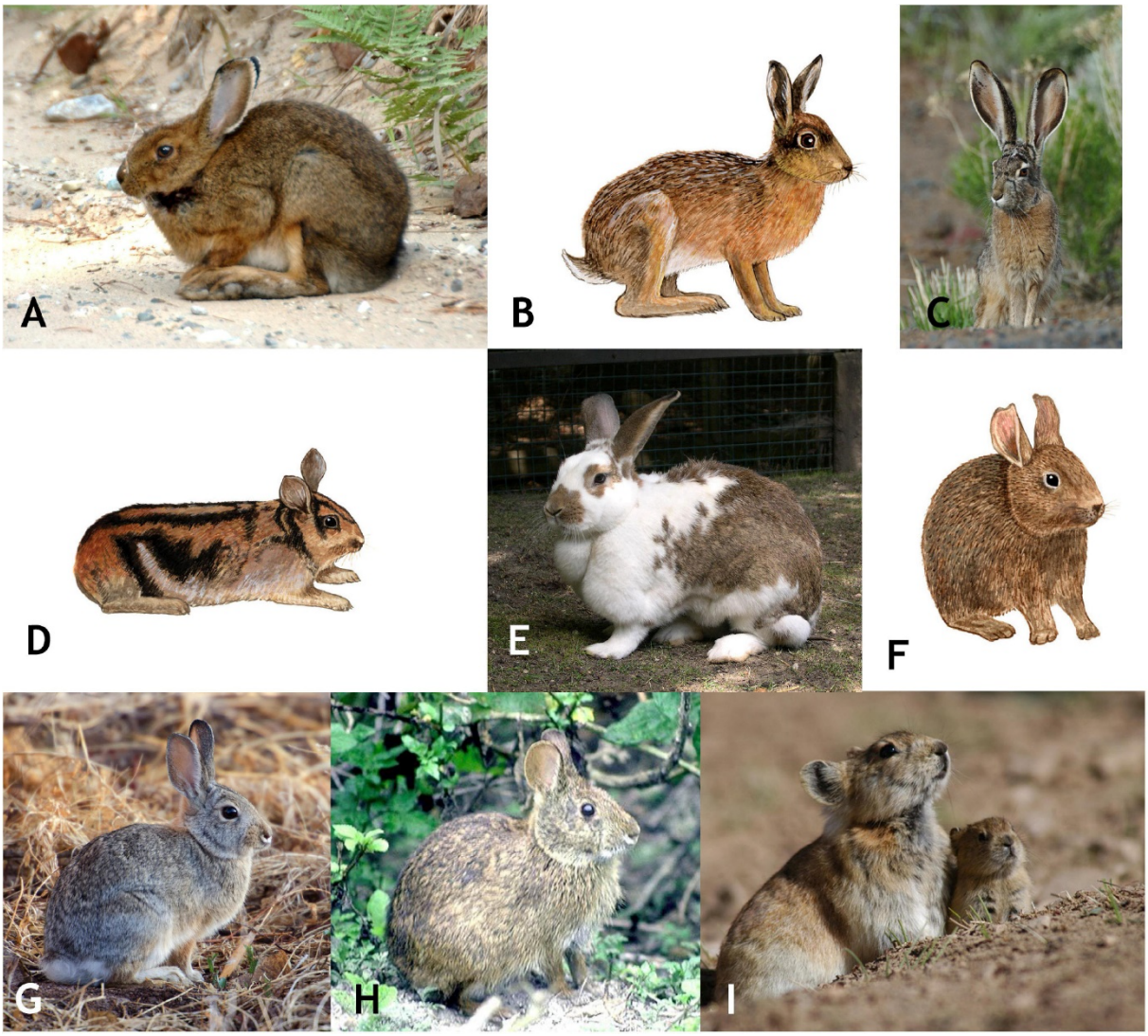


Fig. 2 – A representative selection of extant lagomorphs, including: **(A)** *Lepus americanus* (snowshoe hare); **(B)** *Lepus europaeus* (European hare); **(C)** *Lepus californicus* (Black-tailed jackrabbit); **(D)** *Nesolagus timminsi* (Annamite striped rabbit); **(E)** *Oryctolagus cuniculus* (European rabbit); **(F)** *Romerolagus diazi* Volcano rabbit); **(G)** *Sylvilagus audubonii* (Audobon's cottontail); **(H)** *Sylvilagus palustris* (Marsh rabbit); **(I)** *Ochotona curzoniae* (Black-lipped pika). All images from Myers *et al*. (2020).

The first lagomorphs evolved during the last period of the Mesozoic era, dated back to approximately 53 million years ago and are found in the same clade of rodent and primate (O'Leary *et al*., 2013; Fontanesi *et al*., 2016).

However, according to fossils, lagomorphs and rodents have followed an independent evolutionary path and the distinguishable ancestral forms of the two orders are dated around 50 million years ago (Eocene) (Trocchi and Riga, 2005) (Figure 2).

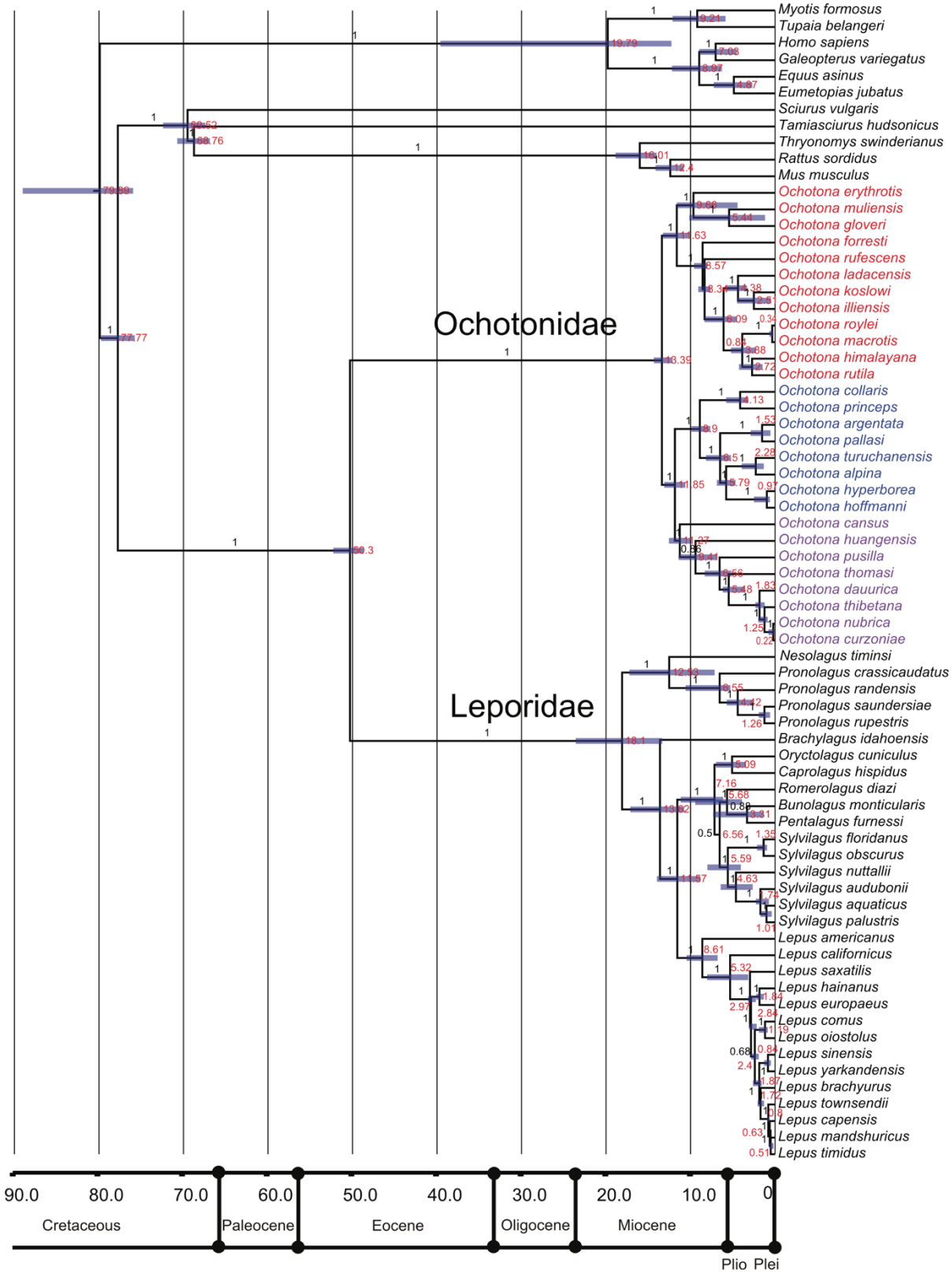


Fig. 3 – Phylogenetic relationships and divergence times of Lagomorpha. Branch labels on the tree give posterior probabilities. Node labels give the median value of divergence time. Blue bars give 95% interval confidence of divergence time. Three ecotype groups of Ochotona are marked in different colors: red, the Mountain group; blue, the Northern group; pink, the shrub-steppe group (Ge *et al*., 2013)

The order Lagomorpha (synonym: Duplicidentata, Illiger 1811) was initially phylogenetically merged with the order Rodentia (synonym: Simplicidentata, Tullberg 1899) until Gidley, in 1912, proposed a separation of the clade into two orders through a new and more precise description (Gidley, 1912).

Despite their origins and global (Holarctic) distribution, the diversity of lagomorphs is relatively small compared to other groups of mammals and, in particular, to rodents. Currently, it is limited to 90 known species, while rodents count over 2000 species (Fontanesi *et al*., 2016; Delaney *et al*., 2018).

At the taxonomic level, the lagomorph appears to be one of the most complex groups. In its complexity, the genus *Lepus* is, in fact, a recently separated genus, whose number of species, which are believed to be part of it, has fluctuated from 71 in 1900, reducing to 21 in the 1960s, and stabilizing at the current 33 (Niethammer and Krapp, 2003). The number of taxonomic characters that can be used to identify the different species is increased thanks to the opportunity of comparison with fossils (Asher *et al*., 2005; Kraatz *et al*., 2021; Ruf *et al*., 2021).

Currently the order is divided into two families: Ocotonidae and Leporidae. The family Ochotonidae comprises the pikas, including one extant genus *Ochotona* and 30 currently recognized species (Hoffman and Smith, 2005). More than 30 extinct genera have been identified as far back as the Eocene, one of which, *Prolagus*, went extinct in the late 18th century (Dawson, 1969; Ge *et al*., 2012). Today, Ochotonidae represents approximately 1/3 of lagomorph diversity (Smith, 2008). Their range is primarily in Asia although there are two North American species, American pikas and collared pikas (Smith *et al*., 1990).

Concerning the family Leporidae the classification proposed by Gidley in 1912 was initially confirmed by Dice in 1929, recognized by Cobert in 1983 and still accepted today by the scientific community which, through increasingly in-depth observations and studies, managed to better characterize the differences between the two groups (Figure 3) (Ge *et al*., 2013).

This family includes 11 genera and over 60 species (Figure 1) and is represented in Italy by three genera, *Lepus*, *Oryctolagus* and *Sylvilagus*, and six species *Lepus europaeus*, *L.*

corsicanus, *L. timidus*, *L. capensis mediterraneus*, *Oryctolagus cuniculus*, *Sylvilagus floridanus* (Lavazza *et al*., 2001; Pierpaoli *et al*., 2003; Smith *et al*., 2018).

Economically and scientifically speaking, lagomorphs are one of the most important groups of mammals. The domestic rabbit for example, is among the major food resources in many countries as well as a key subject in medical research laboratories (Kaya *et al*., 2022). In addition, they are often considered of great value as a game species but at the same time as a "plague", a source of great concern on an agricultural level; for these reasons, this group has led to generous funding for research programs around the world (Alves and Hackländer, 2008).

Given its medium size and its position in the food chain as prey, this group of herbivores plays an important role in the ecosystem; moreover, the diversity in terms of size, behavior, abundance and reproductive fitness allows it to have different ecosystem functions (Fontanesi *et al*., 2016). Some species may, in fact, be rare or so small in number that they are not relevant to the ecosystem but, at the same time, can show an intrinsic conservation value as a rare or vulnerable species (Delibes – Mateos *et al*., 2011). This order includes some of the most endangered mammal species, and according to some studies, the 25% of lagomorphs are recognized as endangered and listed in one of the categories on the IUCN red list (Smith, 1974; Alves and Hacklander 2008; Leach *et al*., 2015).

In particular, among the species of the genus *Lepus*, according to the IUCN, the 21% of these are in a category between vulnerable, threatened or endangered (Table 1).

	1988	1990	1994	1996	2008	2016* 2018
Species						
Lepus alleni				LR(lc)	LC	LC
Lepus altamirae				-	-	-
Lepus americanus				LR(lc)	LC	LC
Lepus arcticus				LR(lc)	LC	LC
Lepus brachyurus				LR(lc)	LC	LC
Lepus californicus				LR(lc)	LC	LC
Lepus callotis			I	LR(nt)	NT	VU
Lepus capensis				LR(lc)	LC	LC
Lepus castroviejoi				VU	VU	VU

Lepus comus				LR(lc)	LC	LC
Lepus coreanus				LR(lc)	LC	LC
Lepus corsicanus				NE	VU	VU
Lepus europaeus				LR(lc)	LC	LC
Lepus fagani				DD	DD	LC
Lepus flavigularis	E	E	E	EN	EN	EN
Lepus granatensis				NE	LC	LC
Lepus hainanus			K	VU	VU	EN
Lepus habessinicus				LR(lc)	LC	LC
Lepus insularis			R	LR(nt)	NT	VU
Lepus mandshuricus				LR(lc)	LC	LC
Lepus nigricollis				LR(lc)	LC	LC
Lepus oiostolus				LR(lc)	LC	LC
Lepus othus			K	LR(lc)	LC	LC
Lepus peguensis				LR(lc)	LC	LC
Lepus saxatilis				LR(lc)	LC	LC
Lepus sinensis				LR(lc)	LC	LC
Lepus starcki				LR(lc)	LC	LC
Lepus timidus				LR(lc)	LC	LC
Lepus tibetanus				NE	LC	LC
Lepus tolai				NE	LC	LC
Lepus townsendii				LR(lc)	LC	LC
Lepus victoriae				LR(lc)	LC	LC
Lepus yarkandensis				LR(nt)	NT	NT*

Tab. 1 – Chronology of the threat status of the 33 species of the genus Lepus as indicated by the IUCN red list. For the years 1994 and earlier: E = Endangered; R = Rare; I = Indeterminate; K = Insufficently Known. For 1996 and later: NE = Not Evaluated; EN = Endangered; VU = Vulnerable; LR (nt) = Lower Risk, Near Threatened (NT = Near Threatened became an independent category in 2001 with version 3.1 of the Red List Categories); LC = Least Concerned; DD = Data Deficient.

In addition, some species distribution models that use variables related to habitat, climate or topography suggest that more than half of the species among this order have been or will be impacted by past and future climate scenarios. This vulnerability shows how much these animals can be of interest, not only because they are potentially in danger or close to extinction but also because they can represent a conceivable bioindicator (Leach *et al*., 2015).

1.2 Origins and Evolution of the genus *Lepus*

Although they are one of the most ancient mammal groups, lagomorphs do not seem to have generated a great multiplicity of "morphotypes", nor have they developed particular adaptations. Conversely, they maintained different characteristics and primitive synapomorphies (Gidley, 1912; Kraatz *et al.*, 2021). The two genera, *Lepus* and *Oryctolagus*, are considered autochthonous to the Palearctic region, that is, originated in the northern hemisphere of the old continent and, as such, are also considered autochthonous in our peninsula (Spagnesi *et al*., 2002).

On the other hand, the genus *Sylvilagus* is native to North America and, even though it now belongs to the zoocoenoses of the European continent, it is considered allochthonous since it was imported from the Nearctic region. The species *S. floridanus* has been widespread in Europe, mainly in Spain and France, but also in Italy, where it was introduced for the first time in 1966 and seems to be naturalized only in the Piemonte region (Lavazza *et al*., 2001; Bertolino *et al*., 2011; Tizzani *et al*., 2020). Despite today the distribution areas are consistently extended, the three genera showed genetic differences in terms of the number of chromosomes (48 *Lepus* - 44 *Oryctolagus* - 36 to 52 *Sylvilagus*) (Bonvicino *et al*., 2015; Smith *et al*., 2018) (Table 2).

Common Name	**Scientific Name**	**Native**	**Introduced**
European rabbit *	*Oryctolagus cuniculus*	SO Europe	Worldwide
European hare / Common hare *	*Lepus europaeus*	Europe and Asia	Sweden, Great Lakes area USA/Canada, New Zealand, Australia, South America, Ireland, Elba, Corsica, Aegean islands
Italian hare / Apennine hare	*Lepus corsicanus*	Italian peninsula, Sicily	Corsica
Iberian hare / Granada hare	*Lepus granatensis*	Iberian Peninsula	Corsica, South France
Sardinian hare	*Lepus* cf. *capensis mediterraneus*	North Africa	Sardinia
Minilepre / Cotton-tailed rabbit *	*Sylvilagus floridanus*	East USA	North America, Italy, France
Snow shoe hare	*Lepus americanus*	Canada, Northern USA and Alaska	North America

Arctic hare	*Lepus arcticus*	NE Canada, Greenland	Newfoundland and Anticosti Island, Quebec
Black-tailed rabbit	*Lepus californicus*	West USA	East USA
Indian Hare / Black-backed Hare	*Lepus nigricollis*	Indian subcontinent	Islands: Indian Ocean and Indonesia
Variable hare / White hare	*Lepus timidus*	Northern Europe, Asia	Faroes, northern islands
White-tailed rabbit / Prairie hare	*Lepus townsendii*	Central West USA, South Canada	Michigan and Wisconsin, USA

Tab. 2 – Lagomorphs introduced outside the areas of origin; * Species considered invasive.

The genus *Lepus* represents the largest and most widespread clade of the Leporidae family, to which the hares properly called belong. They are animals with particular distinctive characteristics such as very developed ears and hind feet, longer than rabbits, great speed of movements and the habit of digging or using depressions already present on the land surface as a refuge and where the leverets are raised (Grzimek, 1973).

This genus is the most representative in the world with 33 ubiquitous species present in the Palearctic ecozone and the Ethiopian region, as well as in North and Central America, Eurasia and Africa (Brooks, 1986; Spagnesi and De Marinis, 2002; Burgin *et al*., 2018). Moreover, some species have also colonized a wide range of biomes, showing their presence in deserts, forests, arctic areas and at different altitudes going from the sea level to the Ethiopian plateau (Alves and Hackländer, 2008; Ferreira *et al*., 2020).

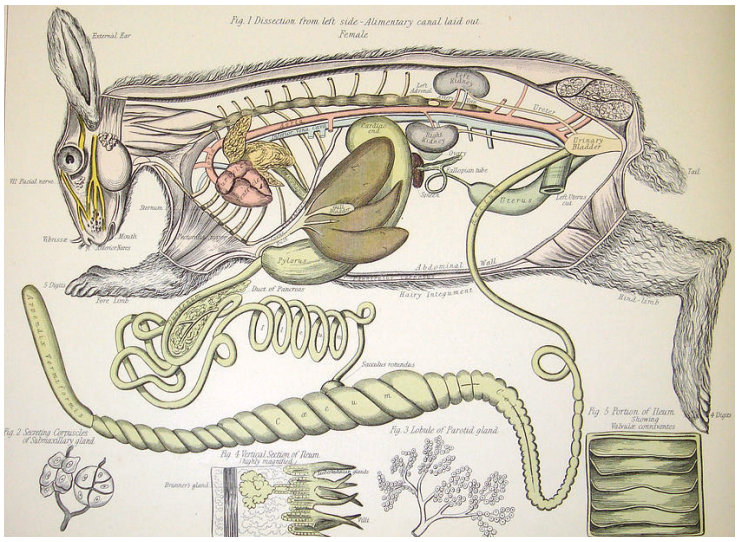
In Europe, colonization, distribution, consistency, and composition of local hare populations followed a combination of biogeographical factors, such as ice expansion and reduction due to glacial and interglacial periods, and anthropogenic factors, mainly through programs of translocation and repopulation (Angelici and Luiselli, 2007). Starting from the late Pleistocene, in the far east regions of Europe, natural events of westward dispersion followed by adaptation toward particular environments and isolation (geographical speciation), contributed to the rising of new species like the mountain hare (*L. timidus*) or the broom hare (*L. castroviejoi*) (Mengoni *et al*., 2015). As regards the brown hare (*L. europaeus*), which shows a major adaptability to a variety of conditions and environments and consequently a wider geographical range of dispersion, the history of the species was further complicated by species turnover due to climate change during interglacial periods (brown hares replaced mountain hares), hybridization and/or mtDNA introgression between different species (mountain hare

and brown hare) and, finally, by relatively recent human activities like translocation of wild individuals beyond its natural distribution area, and the massive release of captive bred hares.

In some countries like Italy, the interest in hunting was, and still is, strongly rooted. The reduction of populations density recorded in different regions was followed by various repopulation programs with the import of specimens from foreign countries such as Romania, Poland or Hungary. However, particularly in Italy, these programs had limited success (Spagnesi and Trocchi, 1992; Angelici *et al*., 2000; Fontana *et al*., 2004; Esposito, 2005a; Angelici and Luiselli, 2007). Anyway, the wide diffusion of this family and the large number of genera and living species, show how this group of mammals still seems to be under evolutionary processes (Grzimek, 1973).

1.3 Anatomy and Behavior

Medium-sized plantigrades with typical characteristics of prey. Slender and elastic body, small head, large eyes placed laterally for a visual coverage of almost 360 degrees but with poor vision, especially in the daytime, given by a non-optimal narrowing of the pupils, and very large ears, longer than the head and with the surprisingly large pavilion that refines the hearing. The legs also highlight their position in the food chain with the more developed hind limbs that have five toes, and are suitable for running and jumping, compared to the smaller and 4-fingered forelegs. The sense of smell is very developed, and the weight varies from species to species in a range between 0.4 and 6 kg.



Anatomy of the hare (Plate XVIII, Anatomy of Vertebrates, 1881).

Lagomorphs' teething of lagomorphs is composed of 28 teeth: they present 2 couples of large, curved, chiseled upper incisors, subjected to continuous wear and growth; 2 couples

of lower incisors; 6 upper and 4 lower premolars; 6 upper and 6 lower molars. As in rodents, lagomorphs do not have canines.

However, three main characteristics differentiate lagomorphs from rodents: the presence of a second pair of incisors, smaller in size and called "pin teeth", placed directly behind the two frontal incisors, the length of the diastema between lower incisors and premolars, which in lagomorphs are greater (Figure 4) and the inability of the forelimbs to grip (Trocchi and Riga, 2005; Alves and Hacklander 2008).

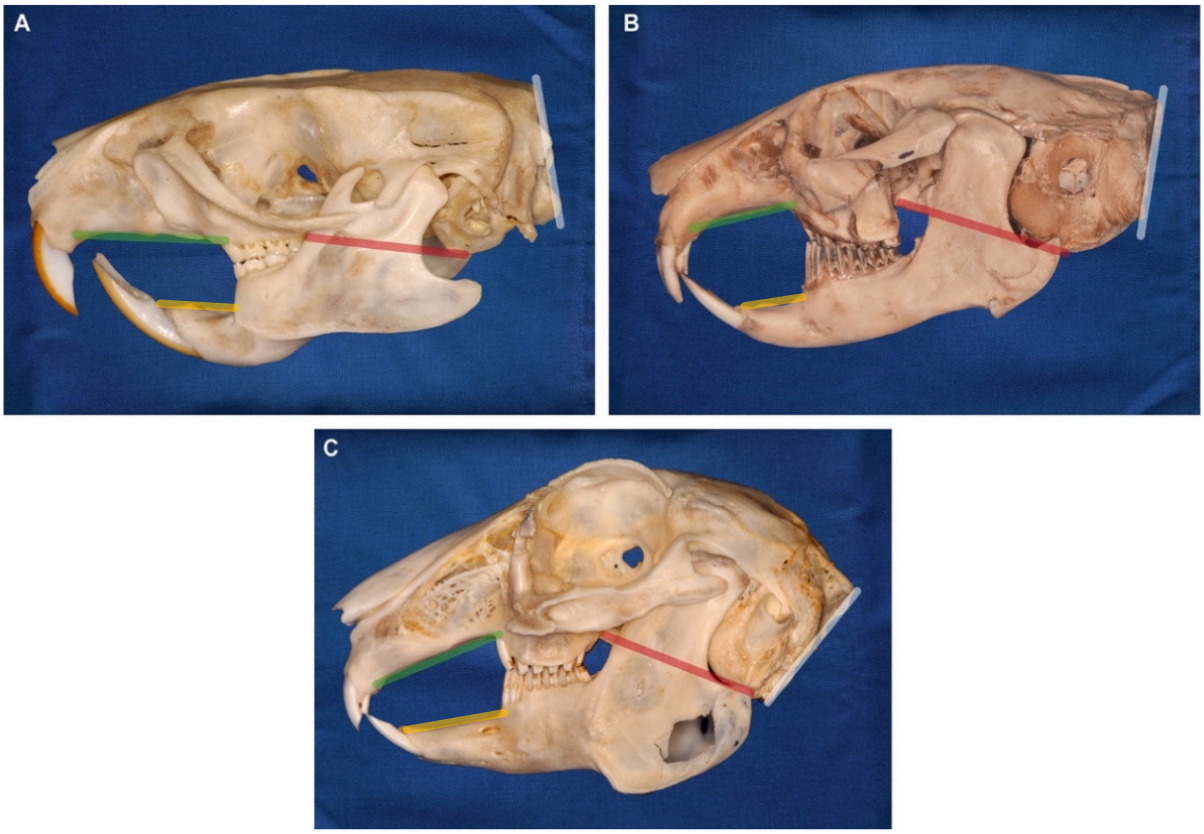


Fig. 4 – Comparison of the skull in rodents and lagomorphs (Ochotonidae and Leporidae). The red line represents the orientation of the basicranium. The green line indicates the palatal plane. The yellow line shows the length of the diastema. The white line shows the orientation of the occiput and foramen magnum. **(A)** Rat (Rodents); **(B)** Pika (Ocotonids); **(C)** Wild rabbit (Leporidae) (Böhmer *et al.*, 2020).

Another peculiarity of lagomorphs is represented by the caecotrophy or "pseudo-rumination". In fact, they have a cecum (Figure 5) where the digestion of the cellulose contained in a particular type of feces takes place after being re-ingested by the animal. These are digested again allowing them to recover good amounts of water, proteins, and vitamins. In general, lagomorphs are not often looking for areas where they can drink but satisfy their water needs mainly through food.

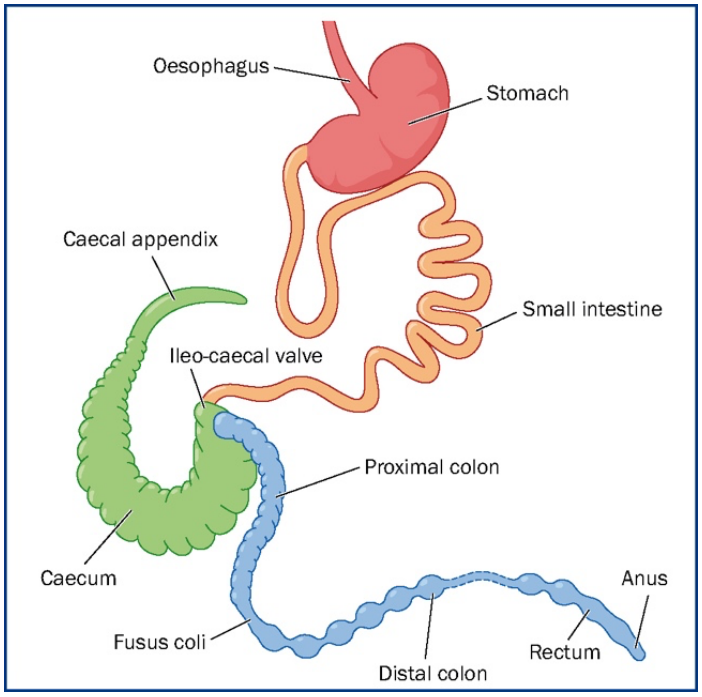


Fig. 5 – Intestinal package of lagomorphs (source: https://www.theveterinarynurse.com/review/article/assisted-feeding-in-rabbits).

Regarding their behavior, they are solitary/antisocial and elusive animals, with crepuscular and nocturnal habits, generally showing great prudence and deep attention to everything surrounding them. Rarely in some populations an “aggregate” type of distribution can be noted, probably because members of the same population can control the area more easily, especially during grazing (Spagnesi *et al*., 1993; Pandini *et al*., 1998). Finally, the last peculiarity concerns their reproduction, even if less observed in nature than in captivity. Females may in fact be subject to superfetation, a particular and rare condition that occurs when two different gestational events, one just begun and the other at the end, overlap (Pandini *et al*., 1998; Roellig *et al*., 2010).

Fig. 6 – Detail of Stroh's Tubercle in adults (ad) and juveniles (juv) (Niethammer and Krapp, 2003).

The age can be estimated through the observation of some morphological characteristics. One of these is the presence of a lateral ossification called Tubercle of Stroh (Figure 6), perceptible on the distal part of the ulna, near the joint of the wrist and showed to the touch in specimens younger than eight months (Trocchi and Riga, 2005; Stankevičiūtė *et al*., 2011; Flis *et al*., 2022).

1.4 Habitat and distribution of European brown hare (*Lepus europaeus* Pallas, 1778)

The European hare (also known as common or brown hare -*Lepus europaeus*-) generally lives isolated and its natural environment is an open territory, with intermediate characteristics between the wooded and desert steppes. It always uses the same paths, which it keeps viable by shortening the plants at the ground level approximately and abandons only when forced to do so (Grzimek, 1973). Burrows are always located at strategic points from where they can always have visual control. In this regard, it has the innate ability to vanish without a trace with particular movements and using paths always taking into account the position of the burrow, the wind and the type of soil (Grzimek, 1973). Even if they are considered sedentary animals, they may be able to make short migrations in unfavorable climatic conditions. Some exception aside, the grazing area is also usually limited close to the burrows, distributed throughout the territory, for a more varied diet (Smith *et al*., 2018). The favorite areas are identified in agricultural patches where, thanks to the abundant and constant availability of food, their growth is favored (Spagnesi and Trocchi, 1992).

Native to the habitats of south-eastern Europe, the Middle East and the Russian plains, starting from the nineteenth century this species seems to have expanded its distribution (Figure 7). This expansion towards the northern regions has been, for most of the last few years, the result of anthropogenic environmental actions (Smith *et al.*, 2018). Deforestation and exploitation of lands for agricultural purposes have, in fact, offered to these animals more favorable conditions for them to spread. It also seems to be one of the most requested prey and, because of its hunting value, it has always aroused such widespread and strong interest that it was imported even in areas where the species was absent: Ireland, Scandinavia and southern Siberia, Far East, regions North America, Argentina, Chile, Australia and New Zealand (Grzimek, 1973; Smith *et al.*, 2018).

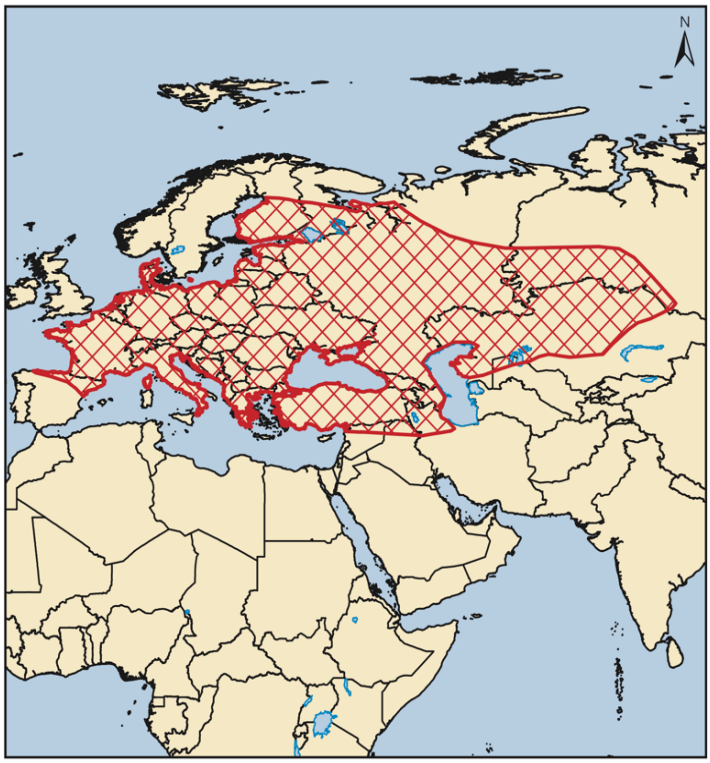


Fig. 7 – Distribution range of the European brown hare in the old continent (Smith *et al.*, 2018).

In Italy, *L. europaeus* was originally distributed in all the northern and central regions and, as a result of repopulation and translocation programs for hunting purposes and the subsequent increase in terms of population size, extended its distribution to almost the entire peninsula (Toschi, 1965). The endemic Italian populations of lagomorphs, which up to 1996 represented the 5.1% of the mammal species in our country, were gradually replaced by populations with mixed genetic traits, so much so that *L. europaeus* species (and probably its native subspecies *L. e. meridiei*) seems to be still present as introgressions in other imported taxa (Amori *et al.*, 1996; Riga *et al.*, 2001; Mengoni *et al.*, 2018). This species was also introduced on Elba and Pianosa Islands, while in Sicily, despite the numerous releases that took place in the last decades and only recently interrupted, no populations of *L. europaeus*

have been identified, and only the species *L. corsicanus* (Trocchi and Riga, 2005; Mengoni *et al*., 2015) can be found.

1.5 Conservation and Management

On a global scale, the conservation and management of lagomorphs involved: (*i*) habitat management, (*ii*) breeding programs, (*iii*) harvesting (in terms of catches for population control), (*iv*) control and (*v*) protection; in particular, the habitat management was the method mainly used to keep the numbers of game animals high (Alves and Hackländer, 2008).

In Europe, as a tradition, deer and hares have always been the most representative mammals for hunting purposes. However, the increasing number of hares due to the numerous restocking programs, and the resultant damage suffered by agriculture in several countries, often made them perceived as "plagues" (Chapman and Flux, 1981). To date, limited hunting seasons, as well as the provision of food during winter have been the main management tools (Alves and Hackländer, 2008).

Among hares, the most worldwide “collected” species are *L. americanus*, *L. californicus* and *L. europaeus*; of the latter, in the 1980s about 70 million specimens a year were exported from South America to Europe (Tume, 2000).

As mentioned above, starting over 2,000 years ago, virtually all over the world and often with undesirable results, numerous repopulation programs have been undertaken. Among the best-known cases there was probably the introduction of European rabbits in Australia, New Zealand and South America, where agriculture suffered severe damage, or the settlement of European hares in Canada, North and South America, Australia, New Zealand and Russia (Alves and Hackländer, 2008).

Similarly, the introduction of *L. europaeus* in Italy seems to have threatened the endemic species, *L. corsicanus* (Trocchi and Riga, 2001).

Concerning control and management, the methods applied since 63 B.C. were and still are aimed mainly at safeguarding agriculture despite the growing interest in protecting these animals (Barrett-Hamilton, 1910; Alves and Hackländer, 2008). Similarly, the protection of the

lagomorphs themselves has very ancient origins, dating back to about 2500 years ago and still today due to their great value as game species of hunting interest. Since 1978, when the Lagomorph Group of the IUCN was established, there has been an increasing interest in those species that were and appeared to be threatened or endangered (Chapman and Flux, 1990).

The ancient relationship between man and hares has meant that the interest in the conservation of these animals and the subsequent creation of programs for improving their natural habitat, grew. Conversely, in the areas where these were introduced, eradication programs exist to limit negative impacts, e.g. hybridization with the Irish hare (the subspecies *L. timidus hibernicus*) (Caravaggi *et al*., 2017). Indeed, in Italy, like in several European countries such as Greece, Spain, France, Germany and Denmark, as well as in other continents like Australia, restocking programs involving the use of animals from other regions have proven to be a threat to regional gene pools.

Thus, to prevent the negative effects of restocking with hares, reared in captivity or non-native, and to encourage natural dispersal, the Italian legislation has promoted the creation of a network of small protected areas (called ZRC "Zone di Ripopolamento e Cattura / Restocking and Capture Zones"), appropriately managed for the "storage" of wild hares used for the repopulation of hunting areas through dispersal or, usually, capture and translocation (Canova *et al*., 2020). Nevertheless, when it comes to the management of natural hare populations the infectious and parasitic diseases to which these animals are subjected is an issue of considerable importance (Pandini *et al*., 1998).

1.6 Main diseases of veterinary interest

Lepus europaeus is susceptible to diseases, and due to its decreasing population size, it is an important research topic (Frölich *et al*., 2001; Wibbelt and Frölich 2005). Several infectious diseases can occur in free-ranging European brown hares and their impact level varies in consequences regarding a single individual compared to a local population. Thus, is of main importance to distinguish between epidemic diseases which influence a whole population and diseases of individuals. Diseases caused by bacteria and parasites dominate while virus infections play a minor, yet threatening role. Spread and extent of infections exacerbate with cool and damp weather (Bock, 2020). For this reason, the knowledge of

diseases in general and a close monitoring of their occurrence will make the prediction of larger epidemics and/or of zoonoses in time, helping to maintain a high species and population health quality as well as a feasible conservation control.

The main diseases that can be observed in these mammals are caused by multiple etiological agents of viral, bacterial and/or parasitical origin and, as part of the health monitoring plans undertaken in a number of Italian provinces and abroad, the attention is focused on typical diseases of hares such as European Brown Hare Syndrome Virus (EBHSV), gastrointestinal and bronchopulmonary strongyles as well as on the surveillance of those with a zoonotic impact like pseudotuberculosis, tularemia and brucellosis (Fraquelli *et al*., 2002).

1.6.1 EBHSV (European Brown Hare Syndrome Virus)

The European brown hare syndrome (EBHS), also called viral hepatitis of hares, is a highly contagious and fatal disease of hares belonging to the species *L. europaeus* and *L. timidus*. The disease was firstly described in Sweden in the '80s and occurred in numerous European countries, hence it is considered today endemic in Europe and Italy (Lavazza and Vecchi, 1989; Lavazza and Cappucci, 1996; Lavazza *et al*., 1996). The causative agent of EBHS is a calicivirus (genus *Lagovirus*), which only causes the disease in hares affecting the internal organs as kidney, spleen and liver. As far as is known, rabbits or other animal species, are not affected. The virus is shed in all secretions and excretions and is very environmentally stable. Transmission presumably occurs directly, particularly faecal-orally or indirectly through contaminated water and feed. The disease is iperacute to acute and is characterized by a very high morbidity and mortality rate (up to 100%). Symptoms are rarely observed in free-living wildlife and they include: weakness, apathy, disorientation, and movement disorders (e.g. paralysis of the hind legs). No therapy is available (Bock, 2020).

1.6.2 RHD (Rabbit Haemorrhagic Disease)

The Rabbit Hemorrhagic Disease is a highly contagious viral disease with a strong spread potential caused by a *Lagovirus* of the Caliciviridae family with an high environmental

resistance. Even though the disease mainly affects domestic and wild rabbits, two different serotypes have been recognized: RHDVa, firstly reported in China in 1984, which has the European rabbit as the only sensitive species (*Oryctolagus cuniculus*) and RHDV2 which is also able to infect some species of hare such as the Italian hare (*Lepus corsicanus*) (Velarde *et al*., 2017; OIE Terrestrial Manual, 2021). In 2010 this new strain of calicivirus, the RHDV2, emerged in France and has been recently reported to cause widespread epidemics not only in European rabbits or in *L. corsicanus*, but also in *L. europaeus*. Symptoms are quite similar to those of the EBHS (Le Gall-Reculé *et al*., 2017; Bock, 2020).

1.6.3 Tularemia

The Tularemia or "rodent plague", is a zoonotic disease due to *Francisella tularensis*, first isolated at the beginning of the last century. Despite some recent cases of tularemia in animals and humans in Australia (Jackson *et al*., 2012; Eden *et al*., 2017), it is a re-emerging disease worldwide with recent outbreaks, which appears to be confined to the northern hemisphere particularly in North America and Eurasia (Maurin *et al*., 2011; Pilo, 2018). Disease carriers are not only *L. europaeus* but also rodents, insect, and birds. Transmission occurs both directly and indirectly (via animal-animal contact, with infected food or bloodsucking insects) and the rapid course leads, in hares, to the death of the infected subject in a few days and more rarely in a few weeks (Mörner *et al*., 1988). Pathoanatomical symptoms are swelling and hyperemia of lymph nodes and spleen. This pathogen can also lead to symptoms in humans which arise after 2-3 days post-infection with fever, headache, sweating and vomiting. Infection occurs mainly after eating poor cooked meat or if hunters get injured during preparation of *L. europaeus* after hunting (e.g., cut in the hand) (Frölich *et al*., 2001; Gyuranecz *et al*., 2010). Generally antibiotic therapy is successful (Bock, 2020).

1.6.4 Brucellosis

Brucellosis is a chronic disease and in hare is mainly caused by the bio-variant 2 of *Brucella suis* of which is the main natural reservoir even if it is limited to some geographical areas since *L. europaeus* is not highly affected and does not represent an important pathogen

for humans unlike the bio-variants 1, 3 and 4. It represents one of the most zoonotic diseases widespread worldwide. The course of chronic septicemia may last for months and in some cases years, appearing to be influenced by factors such as climate, food shortage and / or parasites. The infected individual showed loss of weight, weakens and develops yellow-gray purulent and necrotizing nodular tissue affecting genitals, liver, spleen, lung, and lymph nodes. Infection occurs mainly by mating, but also via an aborted fetus, infected food or suckling (Spagnesi and Trocchi, 1992; Bock, 2020).

1.6.5 Pseudotuberculosis

Pseudotuberculosis is one of the most important diseases which occurs primarily in *L. europaeus* (30 to 50% of individuals losses) and rodents, but can also occur in other mammals and birds, as well as in humans Pseudotuberculosis is widespread in central-northern Europe, but regularly reported also in northern Italy caused by *Yersinia pseudotuberculosis*, a bacterium with high resistance in the environment even if very sensitive to light, high temperatures and drying. The disease has a seasonal pattern with chronic forms that last from few days to several weeks and that rarely evolve into acute forms that lead to death in 3-4 days. Impact is higher under cool and damp weather conditions. Infection occurs via oral uptake of infected food with symptoms as diarrhea, respiratory problems, ataxia and paralysis. Humans can become infected by handling infected carcasses (Lavazza, 1998).

1.6.6 Toxoplasmosis

Toxoplasmosis caused by *Toxoplasma gondii* cause cyst formation in liver, spleen, and intestinal lymph nodes. The definitive host is *Felis silvestris catus* (domestic cat); *L. europaeus* and other animals, as well as humans, are intermediate hosts. When affected, *L. europaeus* shows inactivity, loss of appetite, and lies on the ground. The disease is often acute with subsequent death. Some individuals show catarrhal enteritis and white nodules in the liver (Frölich *et al*., 2001). Humans can become infected by handling infected carcasses or eating unwell cooked meat.

1.6.7 Diseases caused by Trematodes

Cattle liver fluke (*Fasciola hepatica*), and sheep liver fluke (*Dicrocoelium dendriticum*) depend on the presence of certain snail species as intermediate hosts. Infections occur via food uptake (Frölich *et al*., 2001). *F. hepatica* symptoms are emaciation, weakness, and edema. However, on the acute course animals generally show a good nutritional status. Pathoanatomically, bile ducts can be expanded vial-like with walls bloated, and the abdominal cavity can be filled with red liquid. *F. hepatica* is rare in *L. europaeus*, whereas *D. dendriticum* affects *L. europaeus* more frequently especially in sheep-grazing areas (Bock, 2020).

1.6.8 Gastrointestinal strongyles

Gastrointestinal worms (nematodes) of importance are *Trichostrongylus retortaeformis* affecting the duodenum, *Graphidium strigosum* affecting the stomach, and *Trichuris leporis* affecting the cecum. *G. strigosum* inhibits secretion of gastric juices and leads, when parasite burden is heavy, to anemia, emaciation, and increased sensitivity to stress. *T. retortaeformis* symptoms are similar to those of *G. strigosum*. *T. leporis* produces toxic metabolites, leading to intestinal necrosis and in leverets also to developmental disorder and weight loss (Newey and Thirgood, 2004; Coulson *et al*., 2018; Bock, 2020).

1.6.9 Lungworms

Among the parasites affecting the bronchopulmonary system the nematodes belonging to the genus *Protostrongylus* (family Protostrongylidae) are the most frequent and widespread, found in up to 60% of populations and is suspected to be among the main causes involved in the decline of hares' populations. The parasites cause diffuse nodular lesions, bronchitis and inflammation of the bronchial mucosa with cough, dyspnea and nasal discharge, the death of the parasitized subject is however rare (Pampiglione and Canestri Trotti, 1999; Battisti *et al*., 2000). The species *P. oryctolagi* has recently been found in 55% of *L. europaeus* in France (Guitton *et al*., 2016).

1.6.10 Other pathogens and ectoparasites

Additional parasites are fleas (*Ctenocephalides canis*, *C. felis*, and *Spilopsyllus cuniculi*, the rabbit flea): *Haemodipsus lyriocephalus*, sucking hare lice; *Ixodes ricinus*, the castor bean tick; *Ixodes hexagonus*, the so-called hedgehog tick; *Listrophorus gibbus*, the rabbit fur mite; and *Psoroptes communis*, the common scab mite (Frölich *et al*., 2001).

Recently, *Leishmania infantum* infections has been detected in *L. europaeus* in Greece and Spain (Ruiz-Fons *et al*., 2013; Tsokana *et al*., 2016). Leishmaniasis is a vector-borne mammalian disease caused by a protozoan flagellate of the genus *Leishmania*, transmitted by phlebotomine sand-fly species. Although, *L. infantum* seems not to cause clinical disease in *L. europaeus* is an important pathogen for humans and other mammals, thus should be subject for further investigations (Bock, 2020).

1.7 Wildlife bronchopulmonary strongyles

The metastrongyloids are a superfamily of bursate nematodes restricted to certain families of mammals. The group is economically and medically important because many species are significant pathogens of domestic and game animals as well as man (Anderson *et al*., 2009).

The taxonomy of the lungworms has been subject to two contrasting tendencies even though there is now considerable agreement about the major groups which should be recognized. Dougherty (1949, 1951) and others recognized a single family, the Metastrongylidae, which was divided into four subfamilies. On the other hand, other specialists have recognized six families and some other fifteen subfamilies. Today's classification is an attempt to capture the best of both systems, viz. the economy of Dougherty's system and the wealth of sound and detailed morphological information contained in the system proposed by Boev (1975) and Kontrimavichus *et al*. (1976) (Anderson, 2009). Among this superfamily we have 5 families.

The family Protostrongylidae contains a homogeneous group of genera found in ruminants and lagomorphs. The Crenosomatidae seems to be a clearly defined but small family found mainly in carnivores and insectivores. The Pseudaliidae is an archaic group restricted generally to the toothed whales. The most controversial proposal in the current work is the separation of the Filaroididae and the Angiostrongylidae. We believe it will be helpful to restrict the former family to abursate forms with the vulva near the tail end, even though genera intermediate between the families can be shown to exist (Anderson *et al*., 2009).

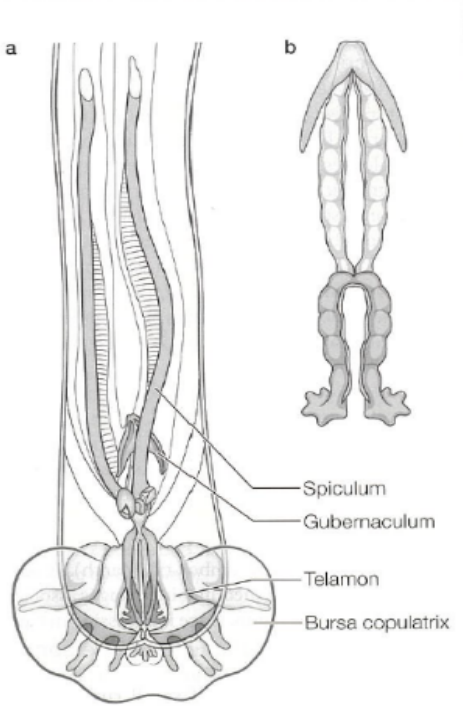


Fig. 8 – (a) detail of the caudal portion of Protostrongilidae adult male; (b) Detail of the gubernaculum

The family Protostrongylidae includes common and important parasites of ruminants and lagomorphs. Its members are transmitted through terrestrial gastropods. The group is distinguished by a highly developed bursa and complex accessory structures, especially the telamon and gubernaculum (Figure 8) spicules are also generally highly developed (Anderson *et al*., 2009).

The family includes 13 closely related genera affecting domestic and wild ruminants, dogs, pigs, horses and 5 species of the genus *Protostrongylus* which are parasites of Lagomorpha (Deplazes *et al*., 2016). Protostrongylids are mostly lung parasites where they release their eggs.

1.8 History and Taxonomy

At the beginning of the 19th century, Frölich first described a pulmonary nematode sampled from a hare naming it *Filaria pulmonalis*. Despite the multiple descriptions of nematode species apparently related to protostrongylids, the history of the study of these organisms is characterized by the lack of “true” and reliable description of the species ending in a taxonomic confusion, posing several doubts about the validity of some of the species described (Boev, 1975).

According to Boev (1975) the study on protostrongylids was divided into three parts.

"[…] This first period of the study of protostrongylids, extending almost up to the beginning of the second quarter of the current century, is characterized by certain "conglomerations" of forms, and accumulation of quite vast literary material without relevant zoological characters of species and hence most of them cannot be considered reliable today […]".

The second period was characterized by a real differentiation of species and by the development of the group protostrongylids. In fact, in 1905, Kamenskii erected the family Protostrongylinae and the genus *Protostrongylus*; between 1927 and 1933, Cameron distinguished some genera such as *Muellerius*, *Aelurostrongylus* and *Elaphostrongylus*; and in 1933 Shul'ts R.S., Orlov I.V. and Kutas A.Ya., were the first trying to classify them, through the observation of the anatomy of the copulatory elements of males (Boev, 1975).

The third period, from the 1940s, deals with the pathogenic effects due to the presence of these parasites together with clinical aspects, epizootiology, diagnostics, therapy and prophylaxis (Boev, 1975).

During the last century, several alternatives were proposed about the systematics of these parasites. At first, the classification of these nematodes was based on morphometrical description of taxonomical characters, but recently, researches based on the molecular analyzes allow to gather new data to evaluate their evolutionary relationship.

1.9 General morphology

Modern research on systematics of nematodes is based on a more and more delicately defined morphology and on increasingly sophisticated morphological studies, as well as utilizing data on the paleontology and biogeography of the host and information on ecology, immunology and biochemistry (Anderson, 2009).

The use of single features to characterize a genus or family, whether that feature is reasonably chosen, is generally inadequate to reflect the real complexity of nematodes relationships. Eventually, only by means of associations of characters as well as the use of

molecular markers can define the taxonomy of genera and their relationships, achieving a more natural classification (Anderson, 2009; Verocai *et al*., 2022).

In 1931 Dikmans described for the first time the species *Orthostrongylus macrotis* naming it *Protostrongylus macrotis*, based on parasites isolated from lungs of a male deer.

Later, Dougherty and Goble (1946) underlined the particular structure of the gubernaculum, described to be different from the structure of the other species of the genus *Protostrongylus*, thus proposing a monospecific genus *Orthostrongylus* to include *O. macrotis.* Finally, phylogenetic analysis based on molecular data combined with the morphological traits of both adult males and females seems to confirm the validity of the genus (Carreno and Hoberg, 1999; Kutz *et al*., 2007).

The morphological analysis was conducted in accordance with the dichotomous keys provided by Baboš (1955, 1961), Boev (1975) and Anderson *et al.* (1992, 2009).

1.9.1 Larvae

With the peculiar exception of Metastrongylidae of Suidae, which use earthworm as intermediate hosts, a general feature of transmission in the Metastrongyloidea is the use of gastropods (usually terrestrial) in which the development occurs to the third and infective stage larvae, as discovered by Hobmaier and Hobmaier (1929) for *Muellerius capillaris* (Anderson, 1992). Many host (notably the artiodactyls) acquire these lungworms from the accidental or deliberate ingestion of gastropods containing infective larvae.

Larvae of protostrongylids at their first-stage of development (L1), are morphologically quite similar to each other. They appear tender and transparent, covered with a double striated cuticle. The mouth opening is terminal and leads into a small buccal capsule, the esophagus is cylindrical and slightly dilated posteriorly and as long as the half of the entire length of the larva. The intestine is granular but, according with Pohl (1960), granules, which are reserve of nutritional material, are different among the species. The posterior part of the body ends in an acute tail.

Based on the structure of the tail two morphological types are distinguishable among larvae of protostrongylids: those with a dorsal spine at the tip of the tail (*Cystocaulus*,

Elaphostrongylus etc.) and those without it, like in the genus *Protostrongylus*. The length of the body can be considered an important diagnostic characteristic, ranging from 0.233 to 0.500 mm while the width from 0.014 to 0.020 mm. The structure of the tail, together with the presence or absence of the aforementioned dorsal spine as well as the length and shape of the spike at the tip of the tail, represents a considerable diagnostic features (Anderson, 2000).

The second stage larvae (L2) still remain undescribed, while the third-stage (L3), the infective stage, are recognized by the presence of two envelopes, one transparent and smooth and the other pigmented and striated. The length varies among species ranging from 0.490 and 0.750 mm. The position of the excretory pore along the esophagus, the distance between anus and tail as well as the morphology of the caudal end, represents useful criteria for diagnosis (Figure 9) (Boev, 1975; Anderson, 1992).

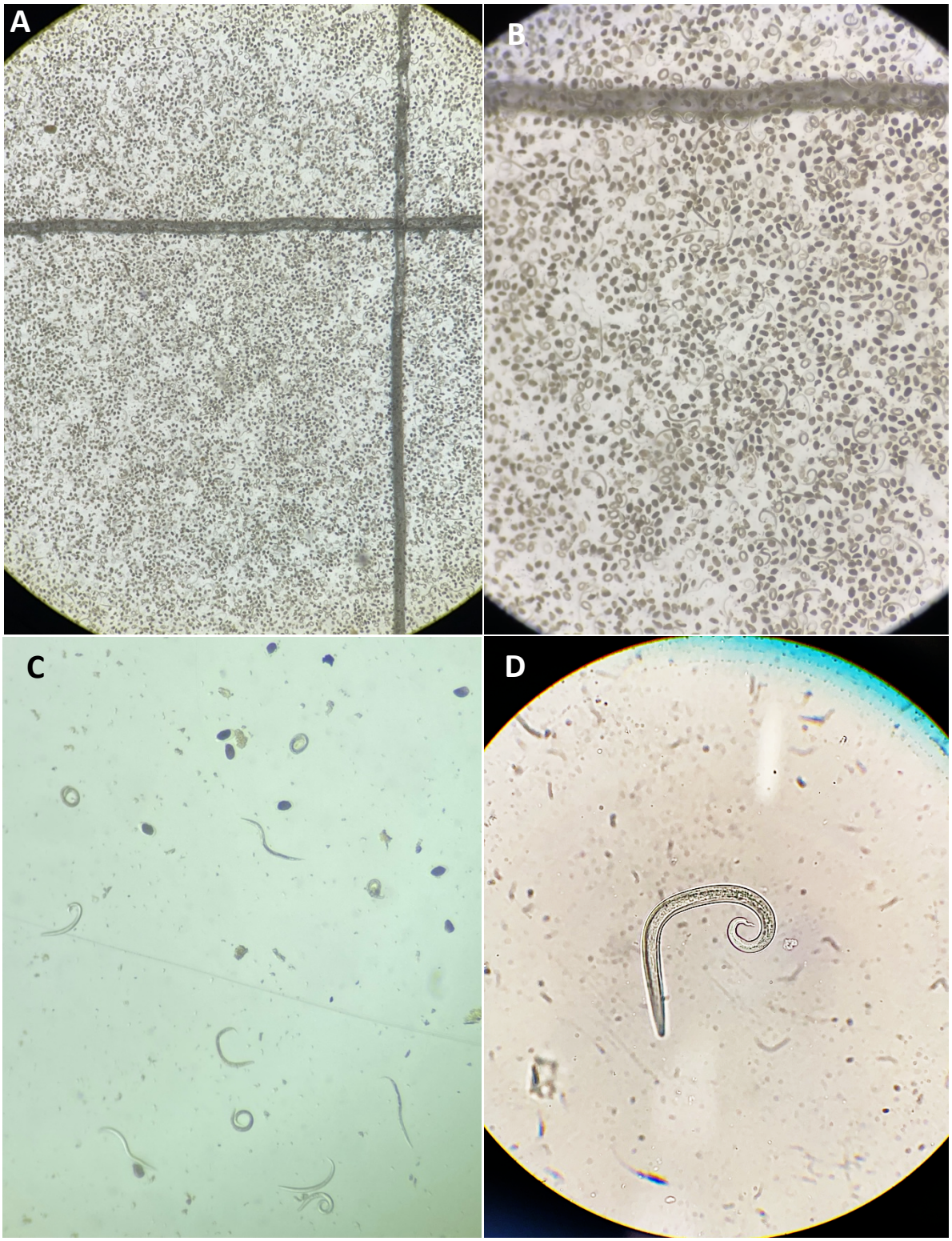


Fig. 9 – Progressive magnification of larvated eggs and larvae of *Protostrongylus oryctolagi*. (A) 0.5 X; (B) 1.5 X; (C) 2.5 X; (D) 4 X.

1.9.2 Males

Males length vary from 5 to 150 mm and between 0.028 and 0.5 mm width. The cephalic end is similar in structure and the mouth is surrounded by a species-specific number of lips (the exact number are still not known). The caudal end showed a genital bursa and its degree of development is similar among protostrongylids.

The bursa consists of a semispherical unpaired dorsal rib, paired externo-dorsal rib and groups of paired lateral and ventral ribs. The caudal end is supported by a skeleton-like structure described by Gebauer (1932) as “arches”. The presence of a developed telamonic apparatus is a constant morphological feature and the degree of development and configuration of individual parts of this structure are important at species level, (Baboš, 1961; Boev, 1975).

Spicules consist in a central column (body) and two alae and are usually equal in length (varying among species from 0.112 to 2 mm); moreover, the apex of the distal end is generally not variable at species level, though sometimes they could be use as a marker for diagnosis. A compact structure near the cloaca is named gubernaculum and it is used, as a muscle, to direct the spicules during copulation. This consist of four parts: “head” (capitulum), “ears ” (lateral branches, according to the terminology used by Schulz, Orlow and Kutass (1933), “body” (corpus), “legs” (crura) and “steps” (pedes) (Figure 10a,b) (Dougherty, 1945).

An atypical shape of the head of the gubernaculum is seen in *O. macrotis* in which the head in dorsoventral position have one end directed proximally and the other distally (Baboš, 1961; Boev, 1975). In this species, adult males and females are the only specimens deeply described. Length vary from 26 to 34 mm for males (35 to 47 mm in females), 0.099 to 0.165 mm width. The head of the gubernaculum is absent, the body and legs usually are situated immediately behind ends of spicules and the latter are proximally fused and pigmented. The bursa is not divided into lobes.

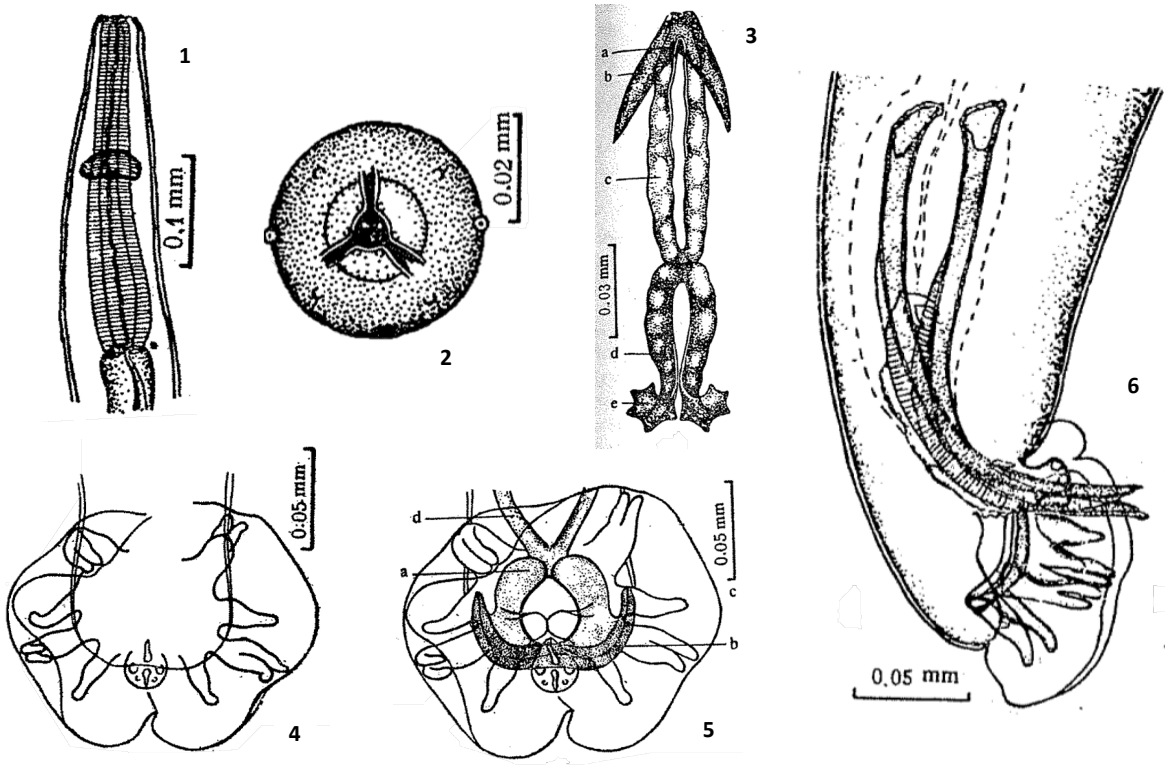


Fig. 10a – Morphology of protostrongylids adult males. 1 – Anterior end of body, lateral view; 2 – Cephalic end, apical view; 3 – Gubernaculum (a – head; b – ears; c – body; d – legs; e – steps / distal ends of legs); 4 – Genital bursa, dorsoventral view; 5 – Structure of bursa and telamon (Plates of telamon: a – ventral; b – basal; c – transverse; d – muscular cords); 6 – Caudal end, lateral view. (Boev, 1975).

Fig. 10b – Detail of caudal end of adult males of *Protostrongylus oryctolagi*. (a) Papille; (b) Head of gubernaculum with ears; (c) Spicules; (d) Aleae of spicules; (e) Legs of gubernaculum; (f) distal end of legs of gubernaculum.

1.9.3 Females

Adult females, as well as larvae, do not possess useful taxonomical characters for their identification, neither within the limits of individual genera nor even in subfamilies, because of their "presumed" morphological monotony (Figure 11a,b). However, some distinctive features have been recognized: these are mainly based on measures of distance, size or shape of specific organs such as the vagina, sphincter and / or provagina. For example, in *Pneumocaulus kadenazii* the size of the vagina (0.237 mm) is smaller than that of the *P. africanus* (2.46 mm) or again, the shape of the sphincter, which generally turns out to be the terminal part of the vagina, joined to the uterus, identifiable *"[…] in the form of a solitary coupling at the place of divergence of the uteri […]*" in species such as *Protostrongylus rufescens*, *P. raillieti*, *Spiculocaulus leuckarti*, *Cystocaulus ocreatus* or also *Varestrongylus pneumonicus*. The presence or absence of a provagina have greater significance in the diagnostic of females. However, different types of provagina (tubular, cowled, ligulate and sheathlike) together with transitional shapes may be found in most protostrongylids but it could also be absent as shown in *Protostrongylus rushi*, *P. terminalis*, *Spiculocaulus orloffi*, *Orthostrongylus macrotis*, and all the elaphostrongylins (Boev, 1975).

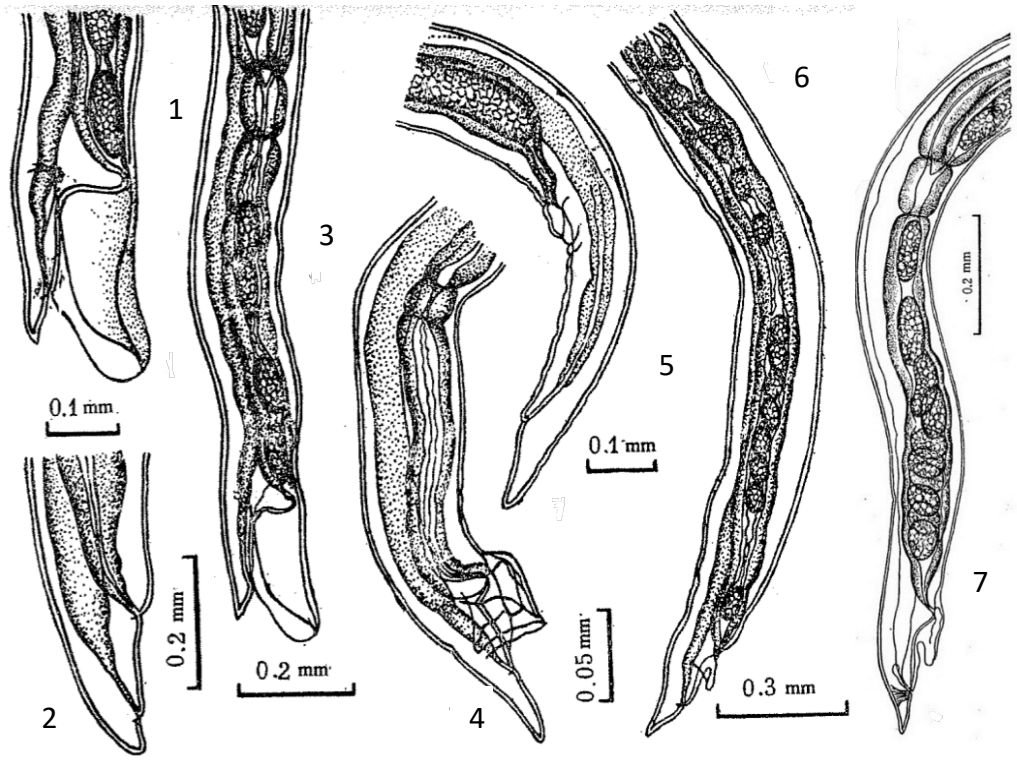


Fig. 11a – Caudal end of female. 1 and 3 – *Protostrongylus raillieti*; 2 – *Elaphostrongylus cervi panticola*; 4 – *Cystocaulus ocreatus*; 5 – *Muellerius capillaris*; 6 – *Protostrongylus hobmaieri*; 7 – *Protostrongylus rufescens* (Boev, 1975).

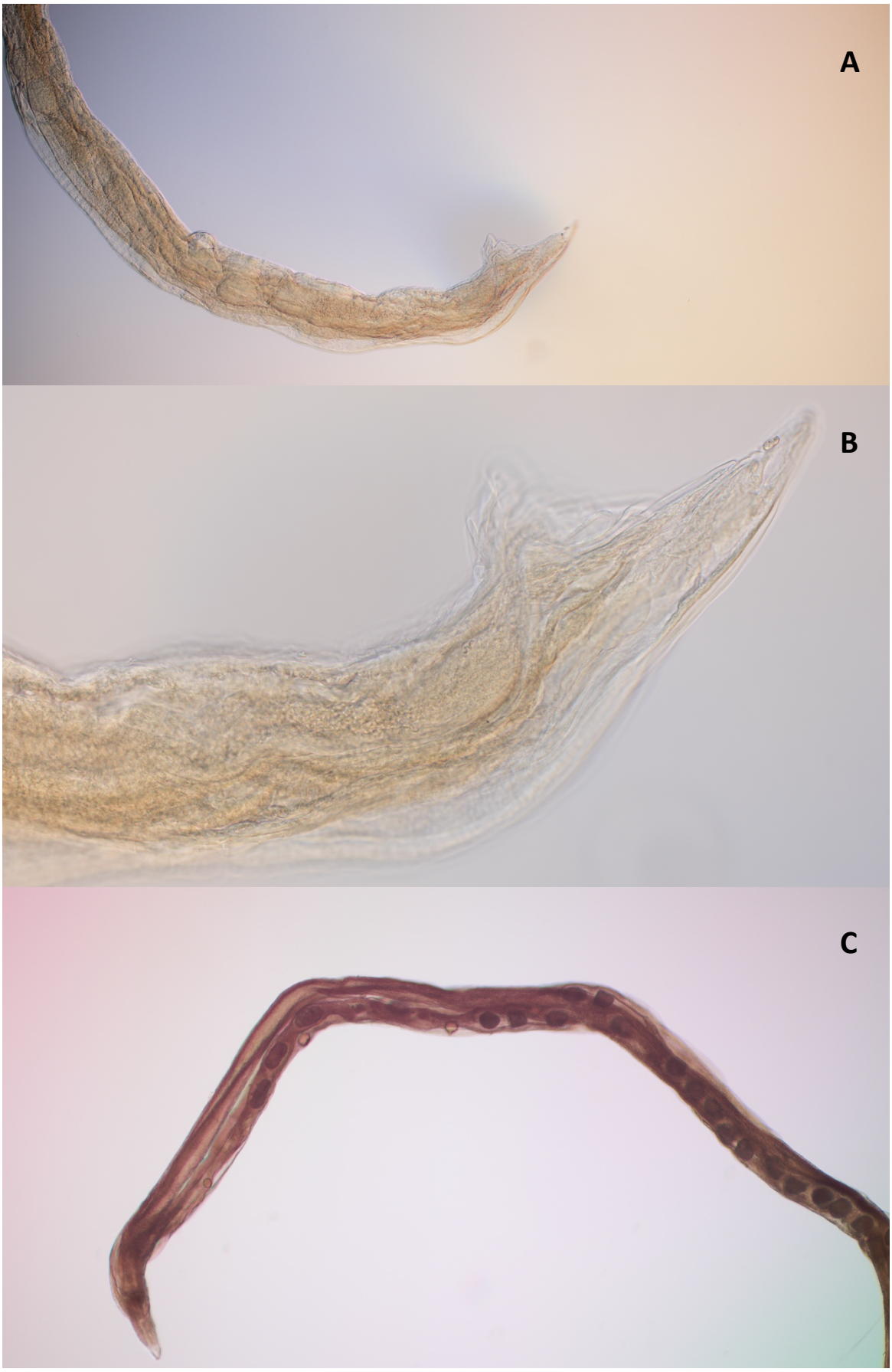


Fig. 11b – Details of the morphology of the caudal end of *Protostrongylus oryctolagi* adult female with particular of eggs in oviduct; (**A**) and (**B**) lateral view; (**C**) dorsoventral view.

1.10 The genus *Protostrongylus* (Kamenskii, 1905)

This genus affects domestic and wild ruminants, dogs, pigs, horses and also lagomorphs which share the genus *Protostrongylus* with the artiodactyls. Since protostrongylids are predominantly parasites of ruminants it has been speculated they have been further adapted to lagomorphs (Anderson, 1982). These are parasites of the vascular, nervous and respiratory systems of mammals (Carreno and Nadler, 2003; Anderson, 2009). The term "bronchopulmonary" indicates the target organ of these parasites, which at the adult stage are located in the bronchi, bronchioles, alveoli or lungs blood vessels. In particular, the latter site is the target of the species of the genus *Protostrongylus*.

These are a group of parasites of great veterinary importance, including about 29 species. Seven of these are described in lagomorphs and, in particular, *Protostrongylus pulmonalis* (Frölich, 1802), *P. cunicularum* (Joyeux and Gaud, 1946) and recently *P. oryctolagi* (Baboš, 1955), were found also in Italy (Guarniero *et al.*, 2022).

1.11 Geographical distribution

Nematodes belonging to the genus *Protostrongylus* (Nematoda, Protostrongylidae) shown an Holarctic distribution and, in particular, they have been described in hares in Hungary (Baboš, 1955), Sweden (Burgaz, 1969), Spain (Casanova *et al*., 1999), Finland (Soveri and Valtonen, 1983; Laakkonen *et al*., 2006), Austria and Czech Republic (Chroust *et al*., 2012), France (Lesage *et al.*, 2014), Poland (Kornas *et al*., 2014), Italy (Costantini *et al*., 1990; Sergi *et al*., 2018; Guarniero *et al.*, 2022), Bulgaria (Panayotova-Pencheva *et al*., 2018; 2019) as well as in the USA (Bookhout, 1971; Keith *et al*., 1985; Kralka and Samuel, 1990) and Iran (Eslami *et al*., 2000; Zafari *et al*., 2022) (Figure 12).

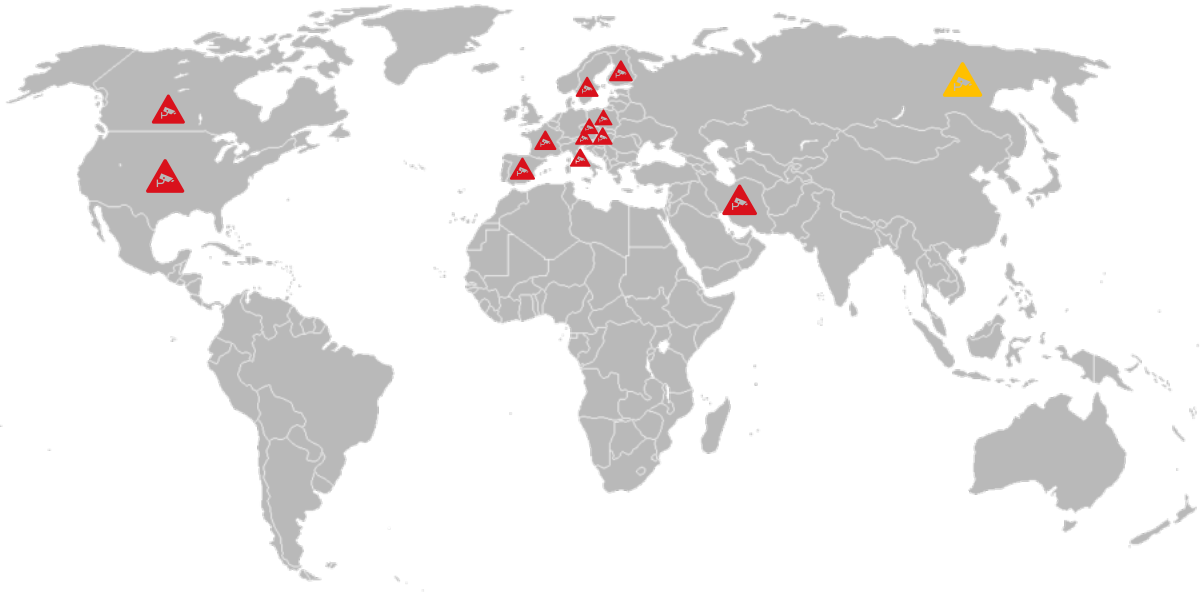


Fig. 12 – Geographical distribution of nematode of the genus *Protostrongylus*.

1.12 Life cycle

Protostrongylus is an heteroxenous parasite, living at adult stage in the lung parenchyma, bronchioles and in small and medium-sized bronchi where females (ovoviviparous) release the eggs which quickly develop into the first-stage larvae (L1). Hatched larvae pass to the pharynx, are swallowed and excreted through the feces which provide them a protective environment in which are able to be viable even for months. In the environment L1 penetrate the foot of coprophagous snails or slugs and moult in a couple of weeks (with summer temperature) into L2 and L3.

The spectrum of intermediate hosts is broad and includes snail's species like *Oxiloma elegans*, *Candidula gigaxii*, *Pupilla muscorum*, *Xeropicta derbentina* or genera like *Helicella, Cernuella, Zebrina, Abida, Bradybaena, Arianta, Theba* and other, less frequently slugs namely *Arion, Agriolimax* and *Deroceras* (Boev, 1975; Sauerlander, 1979; Manga-Gonzalez and Morrondo-Pelayo, 1999; Grewal *et al*., 2003; Lesage *et al*., 2015; Deplazes *et al*., 2016).

Definitive hosts become infected after the ingestion of infected intermediate hosts or plants contaminated by third-stage larvae. L3 larvae are supposed to be actively excreted by the intermediate host during its life or released when it dies. Rarely they have been also

observed to emerge independently from the intermediate hosts and ingested by the definitive host (Anderson, 2000; Kutz *et al*., 2001; Grewal *et al*., 2003).

Once the intermediate host is ingested, larvae are released by digestion and actively invade the intestinal wall reaching the lungs via mesenteric lymph nodes, where they moult into L4, and to the right heart. The L4 penetrate the alveolar walls and establish in the respiratory side of the lungs. The prepatency is species-dependent and last 5-9 weeks. Protostrongylids are long living parasites and may persist in the hosts for several years (Figure 13) (Deplazes *et al*., 2016).

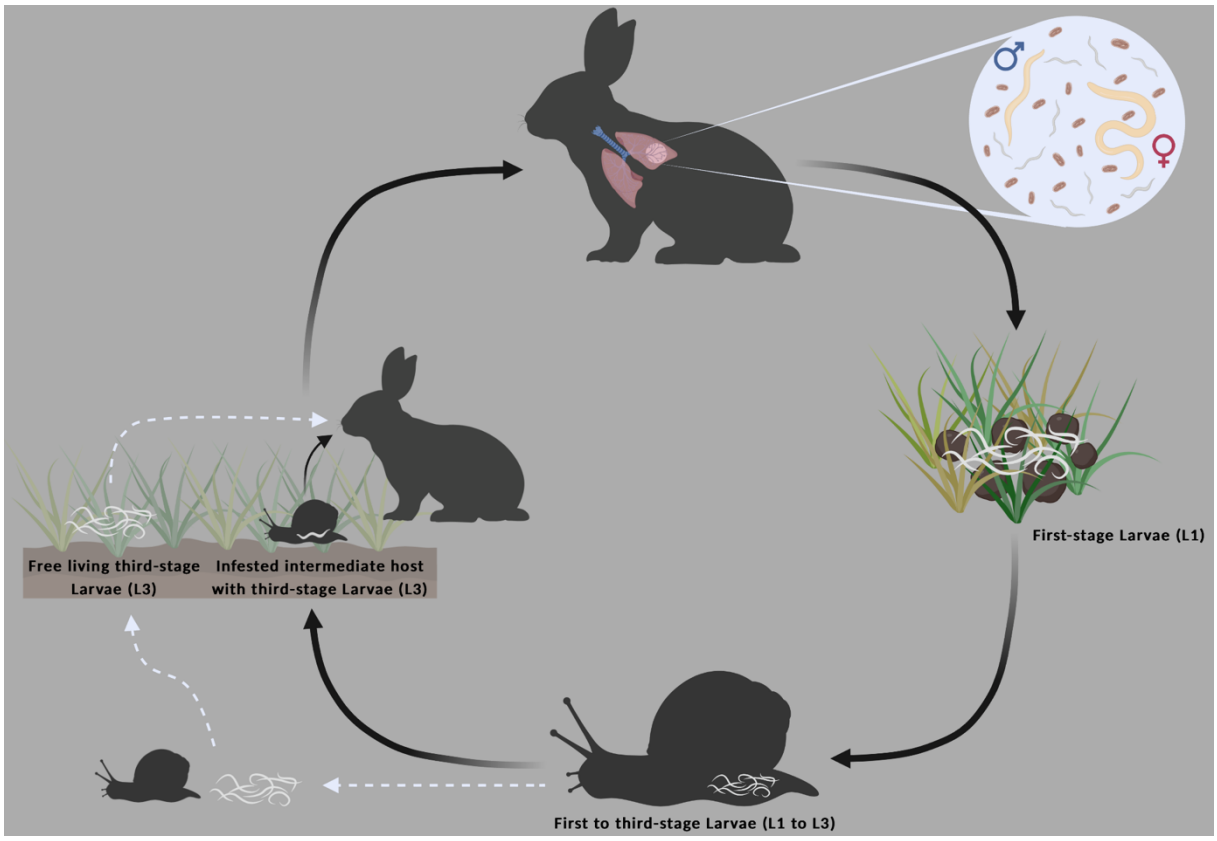


Fig. 13 – Life cycle of *Protostrongylus* spp. in Lagomorphs.

1.13 Species of the genus *Protostrongylus* in lagomorphs

P. boughtoni (Goble and Dougherty, 1943)

This is a parasite of the bronchi, bronchioles and alveoli of snowshoe hare (*Lepus americanus*) in Canada. First-stage larvae were 253—307 µm in length and developed to the

infective stage in the terrestrial gastropod *Vallonia pulchella* in 28—30 days at 18 °C (Kralka and Samuel, 1984a, b).

The first moult took place 14—18 days and the second 28—30 days post-infection. Eggs were noted in the lungs of one hare infected 17 days previously; in other hares the prepatent period was 25—27 days.

Experimentally infected hares passed larvae for 41—104 days. In 12—23 days there was a rapid rise in larval output, followed by a marked decline in the number of larvae passed. The parasite was transmitted successfully to domestic rabbits (*Oryctolagus cuniculus*). Kralka and Samuel (1984a,b) demonstrated that larvae only rarely left snails and that free larvae are not likely to be significant in transmission.

Kralka and Samuel (1990) reported that *Vertigo gouldi* was the major intermediate host in boreal forest habitats in north central Alberta, Canada, where prevalence in hares was 100%. Juveniles became infected within a month of birth and intensities increased to relatively high numbers within 3 months and then declined. There was no evidence of transplacental transmission (Anderson, 2000).

P. cunicularum (Joyeux and Gaud, 1946)

This is a parasite of the bronchi of the wild rabbit (*O. cuniculus*) in Europe. First-stage larvae are 315—370 µm in length. According to Joyeux and Gaud (1946) *Helicella rugosiuscula* was a suitable intermediate host. Infective larvae were 550—750 µm in length. The prepatent period was 26—37 days.

P. kamenskyi (Schulz, 1930) and *P. pulmonalis* (Frölich, 1802)

P. kamenskyi and *P. pulmonalis* (syn. *P. terminalis*) occur together in the lungs of hares (*Lepus* spp.) and rabbits (*Sylvilagus nuttalz*) in Europe and Asia. First-stage larvae are 340-350 µm in length and similar in both species. Several authors studied mixed infections (Ryzhikov *et al*., 1956a, b; Boev, 1975).

Development to the infective stage occurred in the gastropods *Pupilla muscorum* and *Vallonia tenuilabris*, which were considered natural intermediate hosts in the CIS (Ryzhikov *et al.*, 1956). Larvae developed also in *Succinea elegans* and *Vertigo alpestris*.

The first moult occurred in 9-12 days, the second in 20-22 days and larvae were infective in 30—36 days post-infection. The infective larvae were 500—640 µm in length. Some larvae were said to leave the foot of gastropods and could be found on vegetation (cf. Kralka and Samuel, 1990). The prepatent period in hares was 19—22 days.

P. tauricus (Schulz and Kadenazii, 1949).

This is a common parasite of the bronchioles of hares (*Lepus europaeus*) in Europe. First-stage larvae were 340—360 µm in length. Boev (1975) listed the following gastropods as suitable intermediate hosts: *Helicella krynizkyi, H. obvia, Pupilla muscorum, Vallonia costatus* and *V. enniensis*. According to Kadenatsii (1958) larval development in molluscs was completed in 20—25 days at optimal temperatures (presumably greater than 15 °C).

The author believed that larvae which left molluscs in mucus were important in transmission to hares (cf. the observations of Kralka and Samuel (1984a,b) on *P. boughtoni*). Babos (1961) claimed that the first moult occurred in molluscs on day 8 and the second on day 28 post-infection. Infective larvae were 500—540 µm in length. Larvae given to hares appeared in lungs in 12—48 h. The first eggs were deposited in lungs 22 days post-infection and 11—12 days were required for larvae to develop in the eggs.

Rodonaya (1977) reported that *Helicella derbentina* was a suitable intermediate host in Georgia, CIS. Development was completed in this gastropod in 25—30 days; attempts to infect *Enomphalia ravergieri*, *Helix lucorum* and *Vallonia pulchella* failed.

The prepatent period was 40 days according to Rodonaya (1977) and 25—30 days according to Kadenatsii (1958). Infected hares passed larvae for 8—9 months.

1.14 The genus *Orthostrongylus* (Dougherty and Goble, 1946)

The monospecific genus *Orthostrongylus* was proposed by Dougherty and Goble in 1946 to accommodate *Orthostrongylus macrotis* on the basis of some peculiar morphological features of adult males and females distinct to the genus *Protostrongylus* (Dougherty and Goble, 1946; Boev, 1975).

Orthostrongylus macrotis is a protostrongylid lungworm first described as *Protostrongylus macrotis* by Dikmans (1931) based on samples isolated from the lungs of a mule deer from Wyoming. Associated with numerous hosts it was occasionally found in *Odocoileus hemionus* from western North America, mule deer (*O. hemionus hemionus*) and the Columbia black-tailed deer (*O. hemionus columbianus*) (Dikmans, 1931; Landram and Honess, 1955; Worley and Eustace, 1972; Pybus, 1990; Kutz *et al*., 2007). Moreover, *O. macrotis* commonly occurs also in pronghorn antelope (*Antilocapra americana*) and sometimes in moose (*Alces americanus andersoni*) and wapiti (*Cervus canadensis*) (Dikmans, 1932; Landram and Honess, 1955; Honess and Winter, 1956; Boddicker and Hugghins, 1969; Greiner *et al*., 1974; Samuel *et al*., 1976).

The biology and geographic distribution of *O. macrotis* are poorly known.

Fig. 14 – Geographical distribution of nematode of the genus *Orthostrongylus*.

First attempt of a morphological description was carried out by Dikmans in 1931. In its *"Proceedings of the national museum"*, in which he gives a specific diagnosis for this "new" protostrongylid, providing basic morphometrics for adult males and females as follow.

Male: 26 mm long and 165 µm wide. Body smaller immediately anterior to the bursa to about 95 to 100 µm. The esophagus is 440 µm long and 77 µm wide at the base. Spicules are 200 µm long. The spicule sheath extends from the proximal end of the spike to about 10 µm to 15 µm from the distal end. The telamon is usually located immediately adjacent to the terminal portion of the spicules and is somewhat difficult to study. In its general structure it showed similar structures described for other members of this genus. It terminates with two sharp, strongly arched tips. There is no gubernaculum. The bursa, when stretched out, 180 to 190 µm wide and about 160 µm long. The ventro-ventral radius is 25 to 27 µm long, the ventro-lateral 37 µm, the externo-lateral 35 µm, the medio-lateral 46 µm, the postero-lateral 44 µm and the externo-dorsal 38.5 to 42 µm. Chitinous arches are present (Figure 15).

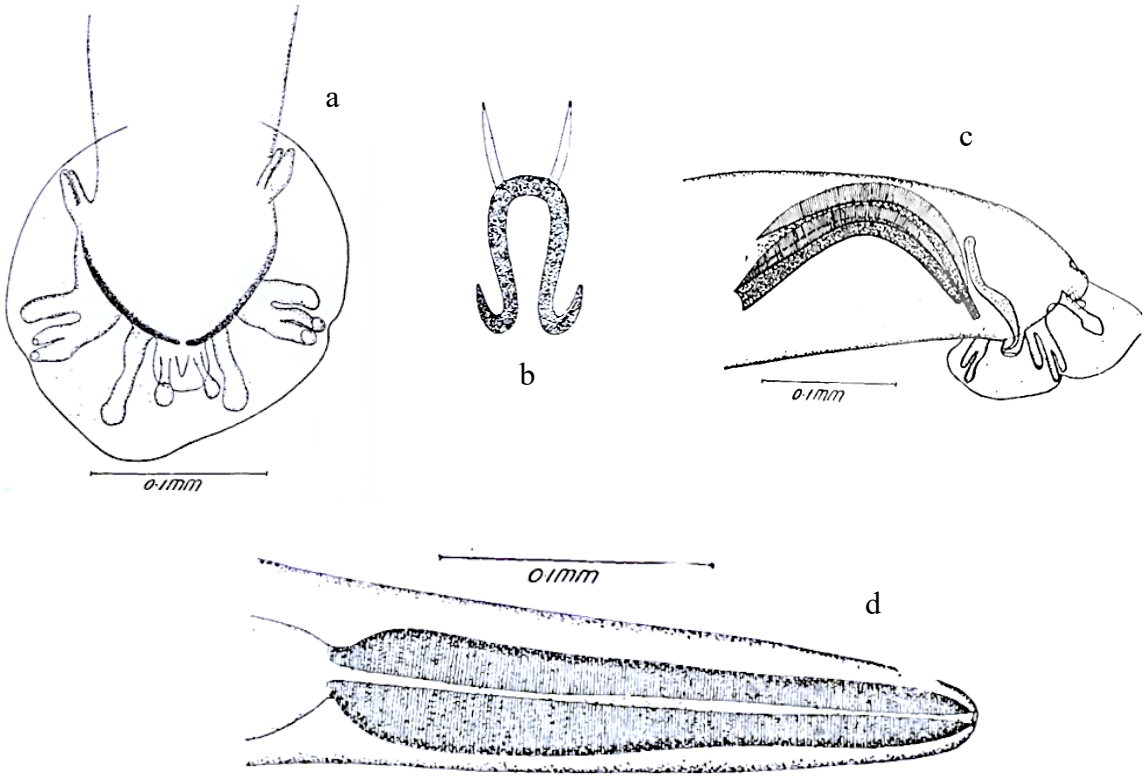


Fig. 15 – Details of *P. macrotis*. (a) Bursa of male; (b) telamon (conceivably referring to gubernaculum); (c) tail end of male ; (d) esophagus (Dikmans, 1931).

Female: 45 to 47 mm long and 190 to 200 µm wide in the region of the vulva. The two uteri unite to forms the vagina, which is 575 to 600 µm long. The vulva-anus distance is 250

to 260 µm, and 110 to 120 µm from the anus to the tip of the tail. This last, ends in a rounded tip. The eggs at the end of the uteri are 57 to 65 µm long and 38.5 µm wide (Figure 16).

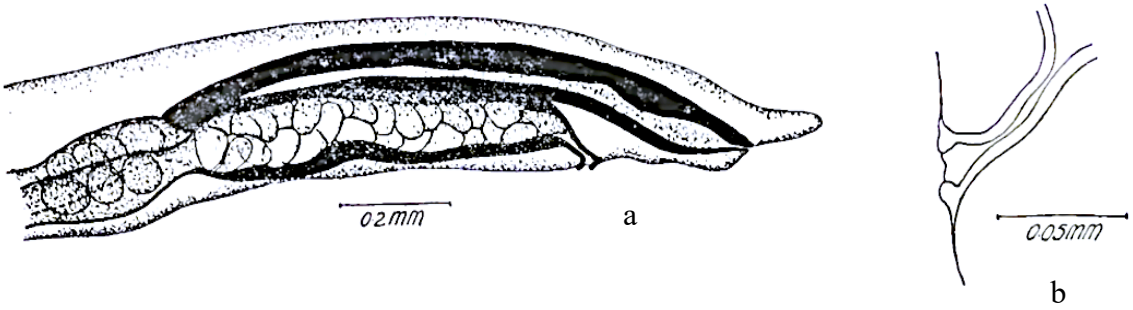


Fig. 16 – Details of *P. macrotis*. (a) tail end of female; (b) vulva (Dikmans, 1931).

In this description, Dikmans (1931) also added a provisional dichotomous key to the *Protostrongylus* species, in which he stated that the species *P. macrotis* does not present the gubernaculum apparatus whether he maybe refers to the absence of the head of the gubernaculum as shown by his attached pictures and later confirmed by Dougherty and Goble in 1946.

First available information on morphometrics and structure of putative first-stage larvae (L1) of *O. macrotis* were provided by Pillmore (1956). However, the identity of L1 examined and reported in his paper, appears to be unsure, as the material cannot be definitively linked to specimens found in *O. hemionus*, *Ovis canadensis*, or other ungulates.

Similarly, the morphologic characteristics of the third-stage (infective) larvae of these species are difficult to study in detail and are superficially similar due to the presence of an enveloping, dark brown, “sheath” which is the modified cuticle of their first-stage.

In 1975 Boev, placed *Orthostrongylus* along with *Neostrongylus* in Neostrongylinae, even if he suggests that the L1 of the former was more similar to those of other protostrongylines.

Knowledge of the diversity and biogeography of lungworm faunas (Protostrongylidae) among northern species of Bovidae has increased with a series of recent studies (Hoberg *et al*., 1995; Kutz, Hoberg, and Polley, 2001; Kutz *et al*., 2001). Moreover, literature on the

diversity of the northern protostrongylid fauna has often been hampered by the paucity of adult nematode collections, which, in the past, has usually been necessary for a definitive diagnosis (Kutz *et al*., 2001).

Recently, Carreno and Hoberg (1999) placed *Orthostrongylus* as sister taxa of *Protostrongylus* based on phylogenetic tree showing unequivocally that individuals belonging to the genus *Orthostrongylus* are morphologically and phylogenetically distant from those of the genus *Neostrongylus*.

Kutz *et al*. (2007) using the first sequence data for *Orthostrongylus*, showed a relationship in an unrooted tree with *Protostrongylus* while exploring broader protostrongylid diversity from North America. However, subsequent phylogenetic studies based on molecular data, however, recognized paraphyly for *Protostrongylus* when *O. macrotis* is excluded (Lesage *et al*., 2014; Kuchboev *et al*., 2015). Conflict in these phylogenetic analyses leaves the status of *Orthostrongylus* unresolved and important information describing the L1 are still unknown.

Nowadays, detailed descriptions of larval stages of *O. macrotis* are still lacking, despite this lungworm has been widely reported, forcing to arbitrarily associate adults with larvae recovered in fecal examination (e.g. Jenkins *et al*., 2005).

In general, the literature on *O. macrotis* is poor, and as a result, its ecology, pathology, and impact on hosts remains unknown. Recently, the evidence in GenBank of a genetically similar nematode sampled from a reindeer from the Taimyr Peninsula, Russia, may have complicated the biogeography of this genus and, by extension, of *O. cf. macrotis* (Loginova *et al*., 2022).

Verocai *et al*. (2022) provided a detailed description of the L1 of *O. macrotis* (Figure 17) by morphological, morphometrical and molecular analysis demonstrating the utility of comparative data for the identification at species level of larval stage along with the necessity of the improvement of archival deposition of adult and larvae specimens (Colella *et al*., 2021).

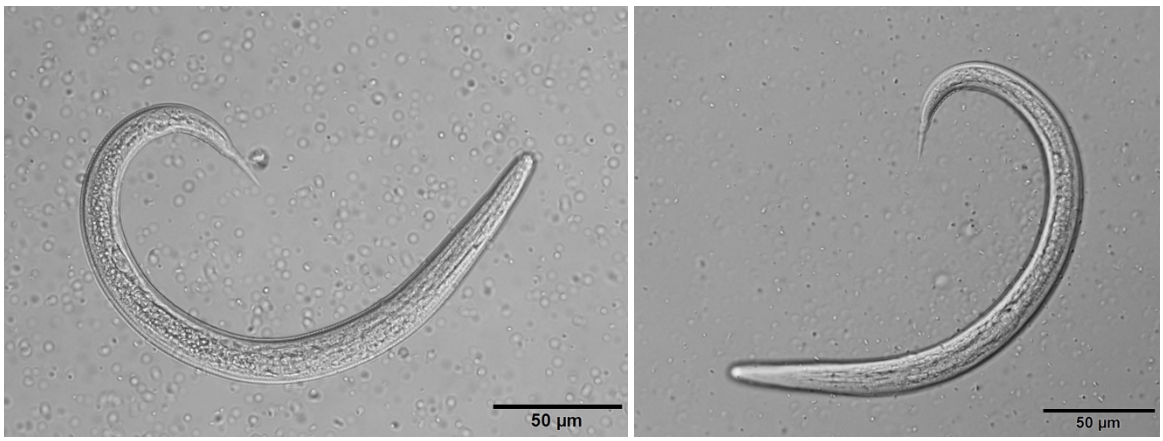


Fig. 17 – First stage larvae of *Orthostrongylus macrotis* recovered from feces of an *Alces americanus andersoni* from Canada.

1.15 Molecular diagnosis

Identification of Protostrongilidae has always represented a real "challenge" among past and present researchers.

During the last two decades, due to the difficulties on the morphological identification of nematodes, many efforts have been made for their molecular identification (Anderson *et al*., 1998; Blouin, 2002; Jenkins *et al*., 2005; Huby-Chilton *et al*., 2006; Kutz *et al*., 2007; Abdel-Gaber *et al*., 2019; Verocai *et al*., 2022). Several studies performed molecular analysis, not only as a support for morphological identification but also in order to investigate the phylogenetic relationships within Protostrongylidae, focusing on markers of nuclear and mitochondrial DNA regions e.g. the small and large subunit of the ribosomal DNA (18S and 28S rDNA), the Internal Transcribed Spacer (ITS rDNA) as well as the cytochrome oxidase subunit 1 (COI) (Gajadhar *et al*., 2000; Carreno and Nadler, 2003; Hoberg *et al*., 2005; Jenkins *et al*., 2005; Asmundsson *et al*., 2008; Ezenewa *et al*., 2010; Bryan *et al*., 2010; Jabbar *et al*., 2013; Kutz *et al*., 2013; Lesage *et al*., 2014; Abade dos Santos *et al*., 2022).

In particular, ITS2 rDNA as well as COI mtDNA are standardized markers for comparison and classification of animals, and moreover, different molecular methods, ranging from fingerprint to sequencing analyses, together with protein-based information, barcoding or even the sequencing of complete mitochondrial genome, have been used to complement morphology-based data.

Eventually, the aim of the modern systematics and researches, is not only the easy and accurate recognition of species but also the identification of their lines of evolution and their affinities in order to reach a deeper understanding of the studied group (Anderson, 2009).

CHAPTER II

Aims

In the framework of the Life Project RESTO CON LIFE (LIFE13NAT/IT/00471) by Life Natura, co-financed by the European Commission between 2014 and 2019, aimed for the re-naturalization of complex island systems, altered by human operations we had the chance to study the endoparasites of *Lepus europaeus*.

Briefly the aim of the Life Project was to safeguard seabirds and nesting avifauna of the Mediterranean shrubland, as well as endemic reptiles, holm oak and juniper woods, coastal dunes, and rocky coast vegetation, temporary ponds, and meadows with annual herbaceous plants.

In particular, action C2 of the aforementioned Life Project, was expected to remove alien species (predators and not) from the Island. One of the target species of this action was the European brown hare (*Lepus europaeus*), introduced for hunting and repopulation purposes between 1920 and 1930 (Angelici and Spagnesi, 2008).

Pianosa Island is located in the Tuscan archipelago (Long. 10°04’44’’ E; Lat. 42°35’07’’ N), about 10 miles from the Elba Island, and extends over a flat area of around 1,030 hectares;

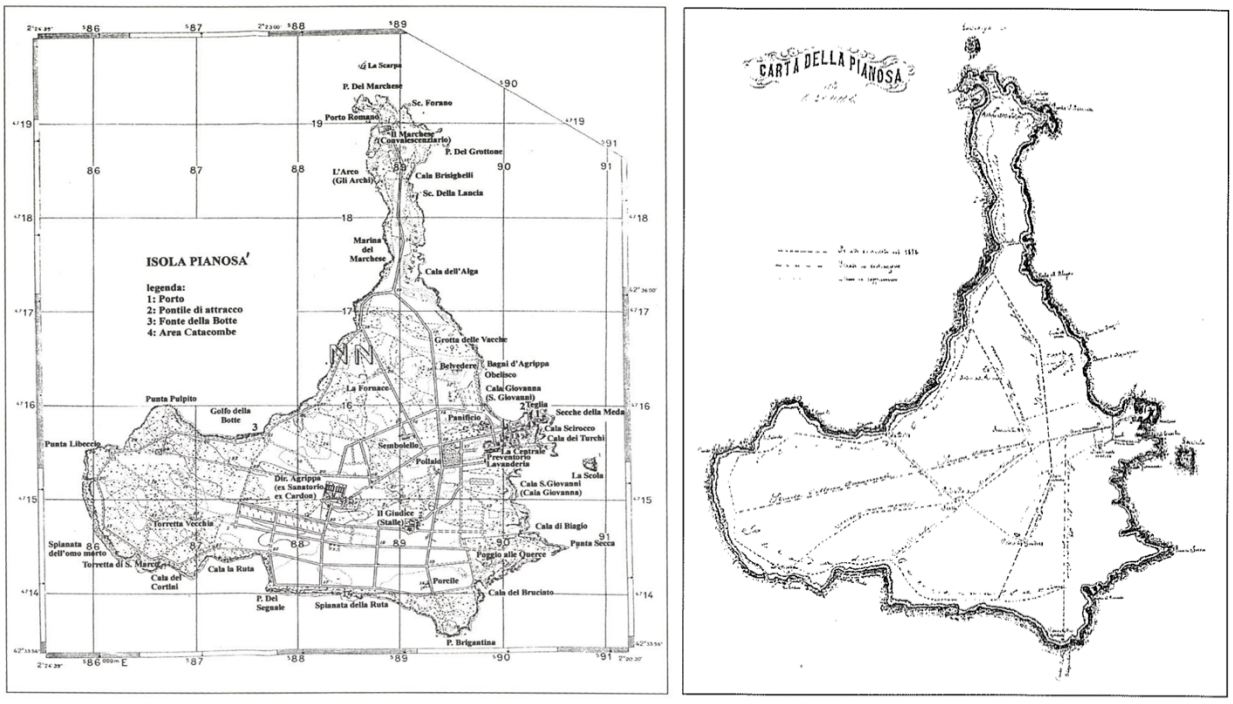


Fig. 18 – Geographic map from the I.G.M.; Plan of Pianosa Island (1874).

since 1997 it has been part of the Tuscan Archipelago Natural Park (PNAT) as well as being included in the Natura2000 European ecological network.

In the island, a Special Conservation Area (ZSC) IT5160016, under the Habitat Directive 92/43/EEC, as well as a Special Protection Area (ZPS) IT5160013, both terrestrial and marine, under the Directive 79/409/EEC have been established as a site of community importance (SIC) on which conservation measures have been applied for the maintenance or restoration of natural habitats and species populations.

Moreover, in the island there is a small population of European hare with about 0,26 hares/hectare, the first documented report of which dates back to 1835 by Repetti (1835) and was subsequently confirmed by Zuccagni-Orlandini (1836) and Simonelli (1884) (Trocchi *et al*., 2019).

Thus, the Life project allowed for a more in-depth study of hare population present in the island. The results of the genetic analyses carried out on hare showed that this population belong to the subspecies *Lepus europaeus meridiei* (Hilzheimer, 1906) and appears to be genetically separated from all other peninsular populations (Mengoni *et al*., 2015; 2018).

Its discovery and the hypothesis of a long period of reproductive isolation is of great value for the biodiversity of the Italian mammal fauna and represents a significant evolutionary unit (ESU) that requires conservation measures.

For this reason, action C2 was reoriented to the conservation of the subspecies *L. europaeus meridiei* which, moreover, appears to be present only in Pianosa (Mengoni *et al*., 2018), and compromised due to the numerous restoking program for hunting purposes (Riga *et al*., 2001; Angelici and Spagnesi, 2008; Canu *et al*., 2012; Rondinini *et al*., 2013).

In the light of an uncommon and extremely interesting situation of a dense, unhandled, and geographically isolated wild European brown hare population, we examined the community structure of their parasites together with their relationship with hosts, to assess if the characteristics of the parasites or the parasites-hosts' association, could be influenced by each other.

The hypothesis was that, these unusual "all in ones" conditions could play a significant role defining not only the helminth fauna dominance and/or codominance structure, but also the host-parasites coevolutive relationship, thus representing an ideal condition to deepen

biogeographic effects on different but strongly linked species and/or populations over time (longitudinally study).

In parallel, the longtime discussed classification of individuals belonging to the monospecific genus *Orthostrongylus* and the chance to observe, measure and genetically examine samples of this rare genus, gave us the opportunity to increase/extend data for an hypothetic taxonomic reassessment.

CHAPTER III

Material and Methods

3.1 Samples collection

In the framework of the life project operations - LIFE13 NAT/IT/000471 project – RESTO CON LIFE "Island conservation in Tuscany, restoring habitat not only for birds" - 26 hares (13 males and 13 females), belonging to the subspecies *Lepus europaeus meridiei* from Pianosa Island, were collected in 2016 and subjected to post-mortem diagnosis.

Concerning *Orthostrongylus macrotis*, fecal samples from moose (*Alces americanus*) were collected by animals handling during field operations near Alberta (Canada) and related to other projects. The feces were frozen until examination (Verocai *et al*., 2020).

3.2 Parasites isolation and identification from hares

Usually, diagnosis of infection and isolation of parasites of interest are based on finding eggs and larvae, present in the feces, using parasitological quantitative and qualitative tests (Verocai *et al*., 2019). However, identification of eggs and larvae at certain life stages as well as of adult females is often very challenging or almost impossible, and this, as already mentioned above, precisely because morphological differences are either subtle or nonexistent. Their identification is therefore carried out in most cases using only the adult males.

Post-mortem diagnostic analysis allows the detection of both lesions and parasites at a macro and microscopic level.

For the diagnosis and sampling of parasites we proceeded as follow.

The cardiorespiratory system was inspected starting from a macroscopic observation of the trachea and heart to finally focus on the main target organ, the lungs. Both trachea and heart were opened lengthwise and macroscopically observed for lesions or adults of endoparasites.

Initially developed by Skirnisson and Kolarova (2008) for the isolation of adult birds schistosome parasites of the intestinal mucosa, the technique used in this study and described below, was later modified and applied by Lesage *et al*. (2014) for the isolation of bronchopulmonary parasites from European hares in France.

The surface of both lungs was first of all observed macroscopically for lesions and/or nodules, typical of the presence of bronchopulmonary parasites of the genus *Protostrongylus*. When found, nodules were incised in order to allow the spillage of parasites and their subsequent sampling.

The lung parenchyma was initially washed with tap water and divided into parts of about 1-2 cm in size, tearing and manually squeezing the tissue, using scissors when necessary.

Fragments were added to the washing liquid, previously collected, and placed in jars with a double seal screw cap to avoid liquid leaking in the next phase of the process.

Jars were hermetically closed and subjected to a vigorous mixing for about 1 minute. After this phase, larger fragments were further washed in tap water and discarded.

The liquid obtained from the several washes was poured into a conical cylinder adding tap water to reach 1L.

Each cylinder was left to settle for about 30-40 minutes after which about half of the supernatant was removed and the total amount of liquid was reconstituted with fresh tap water. This last step was repeated until the supernatant looks clear (Figure 19).

The sediments obtained were examined under a stereomicroscope and parasites were collected and preserved in 70% EtOH and stored at room temperature.

The parasites (739 Males – 232 Females) collected, were separated based on the developmental stages (larvae and adults) and sex, counted and preserved in 70% ethanol for morphological and molecular analyses.

The caudal part of all the isolated parasites was cut and clarified in Amman's lactophenol, and observed by light microscopy. Morphometrical analysis of portions such as the genital bursa (composed of the genital papillae), the gubernaculum (in all its parts) and the spicules was performed following the keys of Boev (1975) and the descriptions of Casanova *et al*. (1999), Lesage *et al*. (2014) and Panayotova-Pencheva *et al*. (2018).

Fig. 19 – Parasites collection process.

3.3 Parasites isolation and identification from feces

Concerning individuals of *Orthostrongylus* sp., L1 larvae were collected from fecal samples of moose by baker Baermann technique following Forrester and Lancaster (1997) and Verocai *et al*. (2013).

Briefly, fecal pellets were placed into an “envelope” formed by folding a piece of vinyl window screen (12 x 12 cm) fixing the open edges onto the beaker walls. The envelope was then submerged in tap water for 24 hr.

Finally, the screen envelop and pellets were removed and the solution was left to sit for 1 hr before removing part of the water and examining for larvae (Figure 20) (Forrester and Lancaster, 1997).

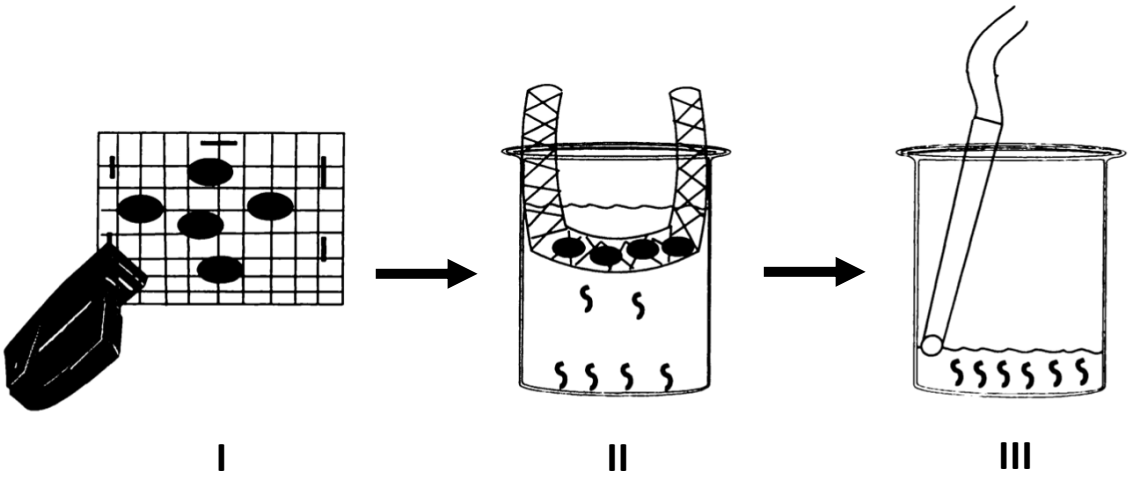


Fig. 20 – Baker method for collecting larvae from feces. (**I**) Single layer pellets contained in screen envelope; (**II**) Envelope placed into a beaker and submerged by tap water; (**III**) Reduced volume of decantated solution with sedimented larvae (Forrester and Lancaster, 1997).

Larvae were subjected to morphological observation, following the keys reported by Dikmans (1931), Dougherty and Goble (1946) and Boev (1975), and to molecular analysis.

Samples were heat-fixed and examined under light microscopy and their morphology was observed through differential interference contrast settings with a X400 magnification. Morphometric data as well as measurement have been compared to already available samples data of other Protostrongylinae, parasites of North American ungulates (Verocai *et al.*, 2022).

3.4 Molecular analysis

For the molecular analysis, the anterior/middle part of the worms was subjected to DNA extraction with the commercial kit NucleoSpin^{TM} Tissue kit (Macherey-Nagel), according to the manufacturer's instructions.

The Polymerase Chain Reaction (PCR) of the whole Internal Transcribed Spacer (ITS) rDNA was performed with 50 ng of gDNA, 10 pmol of each primer NC5 (5'-GTAGGTGAACCTGCGGAAGGATCATT-3') and NC2 (5'-ATGCTTAAGTTCAGCGGGT-3') (Hung *et al.*, 1999), 12,5μl of DreamTaq Green PCR Master Mix (Thermo Scientific) and PCR-grade water up to 25 μl of final reaction volume. The thermal profile consisted in an initial denaturation

step at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 sec, 50 °C for 30 sec, 72 °C for 90 sec, and a final prolonged elongation step at 72 °C for 5 min. The PCR products were run on a 1% agarose gel stained with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific) and sent to the sequencing service StarSEQ (Germany) for sequencing using ABI 3730 DNA Analyzer.

Regarding samples of *Orthostrongylus* spp., the DNA was extracted by the commercial kit DirectPCR Lysis Reagent (Cell) following the manufacturer procedure. The DNA was then subjected to PCR amplification of both ITS2 and 28S rDNA and COI mtDNA with the primers NC1 (5'-ACGTCTGGTTCAGGGTTGTT-3') and NC2 (5'-TTAGTTTCTTTTCCTCCGCT-3') (Kutz *et al*., 2007); C2_f (5'-GAAAAGAACTTTGRARAGAGA-3') and D2_r (5'-TCCGTGTTTCAAGACGG-3') (Lesage *et al*., 2014) for ITS2 and 28S rDNA; LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al*., 1994) for COI mtDNA.

CHAPTER IV

Guarniero I., Bellinello E., Trocchi V., Giannini F., Sulliotti V., and Stancampiano L., 2022. Helminth biocoenosis of *Lepus europaeus meridiei* (Hilzheimer, 1906) from Pianosa island, Italy. *International Journal for Parasitology: Parasites and Wildlife*, 19: 105–109. DOI: https://doi.org/10.1016/j.ijppaw.2022.08.008.

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

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ABSTRACT

Pianosa is a 10 km^2 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 meridei* 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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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 *Davtianostrongylus*. 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 *Protostrongilus* 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^2). 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 hare.

2.4. Molecular analyses

The middle or anterior part of 20 male worms from four different hares was stored at −20 °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 µl of DreamTaq Green PCR Master Mix (Thermo Scientific) and PCR-grade water up to 25 µl of final reaction volume. The thermal profile consisted in an initial denaturation step at 94 °C for 5′, followed by 30 cycles of 94 °C for 30″, 50 °C for 30″, 72 °C for 90″, and a final prolonged elongation step at 72 °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).

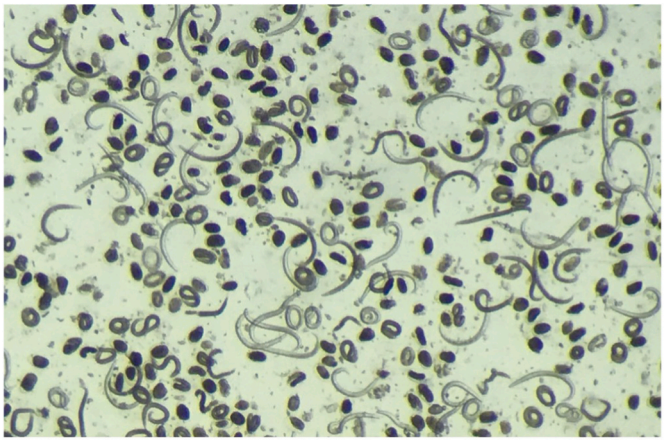

Fig. 1. Larvae and larvated eggs of *Protostrongylus oryctolagi* in lung fluid.

Table 1
Descriptive statistics of main parasitological results.

	Obs	Prevalence % (Maximum prevalence %)	Abundance (sd)	Min-Max	Median
Protostrongilidae larvae	26	100	–		
P. oryctolagi females	20	70	11.60 (18.30)	0–76	3
P. oryctolagi males	20	100	36.55 (44.99)	1–182	21.5
P. oryctolagi 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).

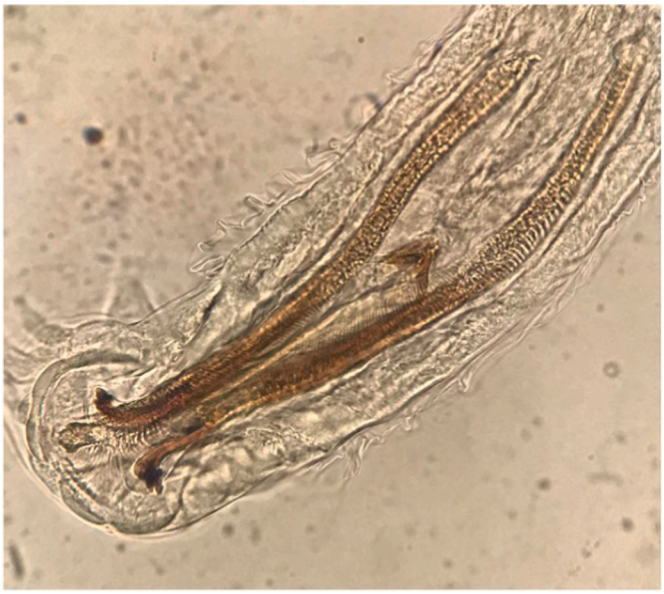

Fig. 2. *Protostrongylus oryctolagi*: tail of adult male.

Table 2
Negative binomial regression model performed on *Protostrongylus oryctolagi* male abundance as dependent variable.

P. oryctolagi	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 *Cernuella* spp. have been identified as possible intermediate hosts in France (Lesage et al., 2014), and *Cernuella 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 accidently 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.

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Declaration of competing interest

None.

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CHAPTER V

Verocai G.G., Kafle P., Sulliotti V., Lejeune M., Hoberg E.P., and Kutz S.J., 2022. Morphometry of first-stage larvae of *Orthostrongylus macrotis* (Nematoda: Protostrongylidae), lungworm of wild ungulates from western North America. *Journal of Parasitology*, 108 (4): 322–329. DOI: 10.1645/22-20.

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MORPHOMETRY OF FIRST-STAGE LARVAE OF *ORTHOSTRONGYLUS MACROTIS* (NEMATODA: PROTOSTRONGYLIDAE), LUNGWORM OF WILD UNGULATES FROM WESTERN NORTH AMERICA

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KEY WORDS

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ABSTRACT

Orthostrongylus macrotis (Dikmans, 1931) is a protostrongylid lungworm in wild ungulates from western North America, including mule and Columbia black-tailed deer, pronghorn, and rarely moose and elk. The lack of morphological data for certain developmental stages of *O. macrotis* and the unresolved taxonomic status of the genus indicate a more detailed morphological characterization of the species is necessary. We provide a detailed description of first-stage larvae (L1) of *O. macrotis* including morphological, morphometric, and molecular data. Species identity was confirmed based on molecular sequence data from the internal transcribed spacer subunit 2 (*ITS-2*) and large subunit (*28S*) rDNA. A fragment of the cytochrome oxidase *c* subunit 1 (*COI*) was also sequenced, followed by the determination of genetic distance and phylogenetic analyses. Integrated data describing L1 of *O. macrotis* contributes to a broader understanding of the parasite fauna of wild ungulates from North America and may be of relevance for a future revision of the genus. Further, we outline information for differentiation among species of North American protostrongylids, with typical spike-tailed L1s, circulating among free-ranging and semi-domestic ungulates.

Orthostrongylus macrotis (Dikmans, 1931) is a protostrongylid lungworm primarily associated with subspecies of *Odocoileus hemionus* from western North America, including the mule deer (*O. hemionus hemionus*) and the Columbia black-tailed deer (*O. hemionus columbianus*) (Dikmans, 1931; Landram and Honess, 1955; Worley and Eustace, 1972; Pybus, 1990; Kutz et al., 2007). Host range is broad and this protostrongylid also commonly occurs in pronghorn antelope (*Antilocapra americana*) (Dikmans, 1932; Landram and Honess, 1955; Boddicker and Hugghins, 1969; Greiner et al., 1974) and infrequently in moose (*Alces americanus andersoni*) (Samuel et al., 1976) and wapiti (*Cervus canadensis*) (Landram and Honess, 1955, Honess and Winter, 1956).

Orthostrongylus macrotis was originally described as *Protostrongylus macrotis* by Dikmans (1931) based on material isolated from the lungs of mule deer from Wyoming (U.S. National Parasite Collection accession, USNPC 30406). Subsequently, the monospecific genus *Orthostrongylus* Dougherty and Goble, 1946, was proposed to accommodate *O. macrotis* based on a suite of unique morphological features of adult males and females considered inconsistent with *Protostrongylus* (Dougherty and Goble, 1946; Boev, 1975); larvae and developmental stages were unknown. Boev (1975) placed *Orthostrongylus* along with *Neostrongylus* in Neostrongylinae, although suggesting that the L1 of the former was more similar to those of other protostrongylines. In phylogenetic reconstruction based on comparative morpholo-

gy, Carreno and Hoberg (1999) placed *Orthostrongylus* as the sister of *Protostrongylus* or as the sister of *Spiculocaulus* + *Protostrongylus*; further, this study unequivocally showed *Orthostrongylus* to be distinct and phylogenetically distant from *Neostrongylus*. Kutz et al. (2007), using the first sequence data for *Orthostrongylus*, showed a relationship in an unrooted tree with *Protostrongylus* while exploring broader protostrongylid diversity from North America. Subsequent phylogenetic studies based on molecular data, however, recognized paraphyly for *Protostrongylus* when *O. macrotis* is excluded (Lesage et al., 2014; Kuchboev et al., 2015). Conflict in these phylogenetic analyses leaves the status of *Orthostrongylus*, and thus *O. macrotis*, unresolved, and fundamental information describing the L1 has remained unknown.

Detailed descriptions of larval stages of *O. macrotis* are lacking, despite this lungworm being widely reported, and it has been necessary to authoritatively link adult nematodes with larvae recovered in fecal examination (e.g., Jenkins et al., 2005). The only available information on morphometrics and structure of putative first-stage larvae (L1) attributed to *O. macrotis* was provided by Pillmore (1956). The identity of L1 examined and reported in this original paper appears to be equivocal, as this material cannot be definitively linked to specimens in *O. hemionus*, *Ovis canadensis,* or other ungulates. Although *O. macrotis* had been collected in samples from deer at Middle Park, Colorado, it is not clear that measurements presented in the paper can be linked to those samples, and more generally the provenance for hosts and localities of those L1 measured for other protostrongyline species remains confused. Pillmore (1956) measured first-stage larvae obtained in bighorn from the Glen Eyrie area, Colorado, which he described as *Protostrongylus rushi* mainly because of its shorter total length; measurements were also given for *Protostrongylus stilesi*. A minimum of 4 species of *Protostrongylus*, however, could have been in sympatry over the areas that were the focus of field collections, and L1 with a sharply tapering tail, lacking a dorsal spine, could not be reliably identified at that time (Pillmore, 1956; Jenkins et al., 2005). Further, Dikmans (1935) also appears to have been in error relative to *O. macrotis* since he demonstrated L1 typical of Protostrongylidae possessing a subterminal dorsal spine (DSL). Such DSL are consistent with genera and species among the Elaphostrongylinae, Muelleriinae, Neostrongylinae, and Varestrongylinae but not the Protostrongylinae (Boev, 1975). Thus, it was assumed that the L1 of *Orthostrongylus* could have a dorsal spine, although there had been no particular basis for establishing an identity for these larvae. Compounding the error, specimens were not archived in a museum collection for reference and confirmation and were not available for further evaluation. Comparative measurements of *O. macrotis* third-stage larvae and those of sympatric species of *Protostrongylus* were later attempted by Kralka and Samuel (1984).

The taxonomic status of *Orthostrongylus* has remained confused due to inconsistencies in the comparative morphology of the L1 and a series of phylogenetic hypotheses based on molecular and morphological data that are not concordant. We resolve the identity and morphology of L1 characterized as *O. macrotis*, providing a detailed description including morphological, morphometric, and molecular sequence data.

Fecal samples of a yearling male moose (*Alces americanus andersoni*) were collected opportunistically (animals handled by biologists for other projects) in the field near Peace River, Alberta, Canada (56°13.56′N, 117°20.34′W) and were kept frozen until examination (Verocai et al., 2020). Spike-tail protostrongylid L1 were isolated from feces using the modified beaker Baermann technique (Forrester and Lankester, 1997; Verocai et al., 2013). Larvae were tentatively attributed to *O. macrotis* based on host and geographic associations, and secondarily on morphology, as this has been the only protostrongyline reported in moose from Canada.

Larvae were heat-fixed (Kafle et al., 2015) and examined under light microscopy with differential interference optics. The morphology of L1 specimens was assessed under bright-field and differential interference contrast (DIC) settings (Olympus BX53 fitted with digital camera, Olympus DP73, Olympus®, Center Valley, Pennsylvania) at ×400 magnification. Photomicrographs and measurements were taken using special software (Olympus© cellSens 1.14 digital software [https://www.olympus-lifescience.com/en/software/cellsens/]).

Genomic DNA (gDNA) was extracted from an individual *O. macrotis* L1 as follows: 50 µl of DirectPCR Lysis Reagent (Cell) containing 25–50 µl Proteinase K solution per 1 ml. DirectPCR reagent was added to each tube and DNA extraction was performed as follows: tubes containing specimens were incubated at 55 C overnight, then at 85 C for 45 min. For species identification, a PCR was performed using primers NC1 (5′-ACGTCTGGTTCAGGGTTGTT -3′) and NC2 (5′-TTAGTTTCTTTTCCTCCGCT -3′) (Kutz et al., 2007); C2′_F (5′- GAAAAGAACTTTGRARAGAGA -3′) and D2_R (5′-TCCGTGTTTCAAGACGG -3′) (Lesage et al., 2014); LCO1490 (5′- GGTCAACAAATCATAAAGATATTGG -3′) and HCO2198 (5′- TAAACTTCAGGGTGACCAAAAAATCA -3′) (Folmer et al., 1994), targeting the *ITS-2*, *28S*, and *COI* regions of rDNA and mtDNA, respectively. PCR amplification was performed in 25-µl reactions containing 10.25 µl of water, 0.625 µl (10 µM) of each primer, 12.5 µl of GoTaq Hot Start Green Master Mix (Promega, Madison, Wisconsin), and 1 µl of DNA template. The amplification conditions used were an initial 2 min denaturation at 95 C, followed by 40 cycles of denaturation at 95 C for 30 sec, annealing at 52.5 C (*ITS-2*) and 50 C (*COI*) for 45 sec, and extension at 72 C for 60 sec, with a final elongation step at 72 C for 5 min. Similarly, the cycling conditions for the *28S* were made of an initial 3-min denaturation at 94 C, followed by 40 cycles at 94 C for 30 sec, 40 C for 60 sec, and 68 C for 60 sec before a final elongation step at 68 C for 10 min. For each run, the final elongation phase was followed by a cooling step to 4 C. Reagent-only reactions were used as negative controls to detect potential contamination.

Editing and molecular and phylogenetic analyses were conducted using ContigExpress (Vector NTI 10.3.0, Invitrogen, Carlsbad, California) and MEGA version 7 (Kumar et al., 2016). BLAST searches were used to compare the resulting sequences to *ITS-2* and *28S* rRNA sequences of *O. macrotis* available in GenBank. Phylogenetic analyses were performed using the Maximum Likelihood method in MEGA 7 (Kumar et al., 2016). The most suitable nucleotide substitution model was estimated as Tamura 3-parameter, Gamma distributed (T93 + G) for *ITS-2* and *28S*. Internal node bootstrap support was determined by 1,000 bootstrap replicates.

Concerning the *COI*, the sequences were compared with the BLAST results, including the mitochondrial genome of specimens

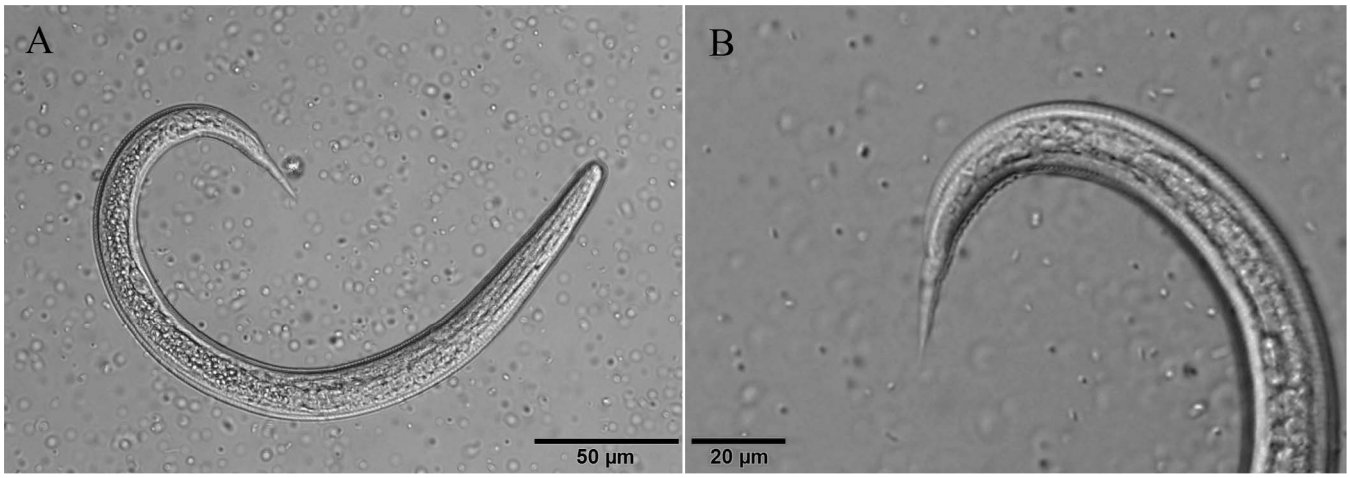

Figure 1. (**A**) First stage larva (L1) of *Orthostrongylus macrotis*, collected from feces of a male moose (*Alces americanus andersoni*) from Peace River, Alberta, Canada. (**B**) Detail of the posterior end of L1 of *O. macrotis*, showing the tail spike.

belonging to the family Protostrongylidae. The best fit model was estimated as Tamura-Nei, Gamma distributed (TN93 + G), supported by 1,000 bootstrap replicates.

The detailed morphometry of *O. macrotis* L1 is shown in Figure 1 and Table I. Morphometric data have been compared to those of Protostrongylinae species known to infect wild ungulates from North America, for which morphological data are available. Measurements are in micrometers (µm), larval specimens examined (n = 20), and the range is followed by the mean ± 1 SD within parentheses.

First-stage larvae: Voucher specimens representing *O. macrotis* isolated from mule deer in Saskatchewan, Canada, were deposited by Kutz et al. (2007) in the U.S. National Parasite Collection, originally held by the Agricultural Research Service, U.S. Department of Agriculture in Beltsville Maryland (USNPC No. 96786, 96787), and later in the Smithsonian National Museum of Natural History (NMNH No. 1391795, 1391796). Specimens in Alberta moose from our study were few and were destructively sampled during analysis. These were determined to be consistent with those in Saskatchewan deer based on sequence identity which represents the original deposition of archival specimens (Kutz et al., 2007).

Sequences produced in this work were deposited in the GenBank under accession numbers OM321430, OM315308, and OM328108. The sequence for *ITS-2* (447 bp) was 99.7% consistent with those for *O. macrotis* produced by Kutz et al. (2007) and was 98.6% consistent with sequences from reindeer in Russia available in GenBank (deposition by O. A. Loginova and S. E. Spiridonov, unpubl. data). Similarly, the sequence for the *28S* region (605 bp) was 99.7% consistent with those from L1 in deer (Kutz et al., 2007) and was 99.5% consistent with the sequences from Russia. Similarity among species attributed to the Protostrongylinae was between 79.8% (62.4–98.9%) and 92.2% (86.9–99.7%), for *ITS2* and *28S*, respectively, when excluding distances between *Orthostongylus* sequences. The partial *COI* sequence produced (698 base pairs) in our current analysis is the first available for *O. macrotis*. All the sequences used as references are available in GenBank (EU018483, EU595592, OL700043, OL700044).

Phylogenetic analyses and genetic distance among protostrongylids based on *ITS-2* show a high similarity among putative populations of *O. macrotis* in Alberta, Saskatchewan, and Siberia; relative to reference sequences (98% bootstrap support). The maximum likelihood trees demonstrate two or three major subclades containing species historically attributed to the Protostrongylinae (56 and 91% bootstrap support) (Fig. 2A). Across this assemblage of genera and species, all analyses reflect paraphyly for *Protostrongylus*, as *Orthostrongylus* and *Spiculocaulus* were included in the clade. Tree topology is consistent with 2 subclades (*Spiculocaulus* + *P. pulmonalis* and *Orthostrongylus* + *Protostrongylus* spp.) or 3 subclades (*Spiculocaulus* + *P. pulmonalis* and *Orthostrongylus* and *Protostrongylus* spp.). Basal stability for these subclades, however, is equivocal, with support of 50% or lower; in contrast, crown subclades showing species relationships are well supported.

Analyses of the *28S* gene diagnose two strongly supported subclades containing species of Protostrongylinae (100% bootstrap support) and are consistent with paraphyly for *Protostrongylus* (Fig. 2B). Concerning the *COI*, no reference sequences were available and the phylogenetic analyses are limited due to the absence of broad taxon sampling. In this analysis, *Protostrongylus* and *Orthostrongylus* are grouped in a weakly supported subclade (Fig. 2C).

Morphological features of L1s attributed to *O. macrotis* confirm structural similarity to larvae described among species of *Protostrongylus*. We confirm the elongate and spike-tail configuration of the caudal extremity, which contrasts with the sub-terminal dorsal spine typical of Elaphostrongylinae, Varestrongylinae, and others (e.g., Boev, 1975; Kutz et al., 2007). The scarcity of morphologic data on L1s of protostrongylines among wild ungulates precludes more conclusive comparisons. Meristic data, however, demonstrate a longer extension of the tail-spike in *O. macrotis* relative to *P. stilesi*, *P. rushi*, and *Protostrongylus coburni* (Dikmans, 1935; Pillmore, 1956; Kutz et al., 2001). Kafle et al. (2015, 2017) have described consistent differences in the dimensions and configuration of caudal extremities among other genera and species of protostrongylids, demonstrating the utility of comparative data in species diagnoses and identification.

Table I. Comparative morphometrics of first-stage larvae of *Orthostrongylus macrotis* and species of *Protostrongylus* associated with wild North American ungulates. All measurements in micrometers (μm). Data in parentheses: mean and standard deviation.

Characters	*Orthostrongylus macrotis** (n = 20)	*Orthostrongylus macrotis*† (n = 5)	*Protostrongylus stilesi*‡ (n = 20)	*Protostrongylus rushi*§	*Protostrongylus frosti*‖	*Protostrongylus coburni*#
Body length	310–366 (327 ± 15.59)	242–256 (243)	342–382 (364 ± 10)	336–371 (350)	—††	400–425 (412.5)
Nerve ring	79–94 (79 ± 4.84)	—	78–93 (87 ± 4)	—	—	—
Excretory pore	76–95 (85 ± 4.89)	59–67 (64)	87–99 (93 ± 4)	96–104 (100)	—	—
Esophagus length	133–153 (142 ± 6.09)	107–128 (115)	136–160 (50 ± 6)	144–152 (148)	—	150–190 (170)
% Esophagus/total length	40–46 (44 ± 1.78)	—	39–43 (41 ± 1)	—	—	—
Esophagus base width	10–16 (12 ± 1.22)	—	—	—	—	—
Max body width	14–18 (16 ± 1.10)	14–16 (15)	15–20 (17 ± 1)	19–20 (19.5)	—	—
Genital primordium	196–240 (211 ± 11.24)	—	206–234 (220 ± 8)	—	—	—
Genital primordium (esophagus-intestinal junction)	53–90 (69 ± 10.46)	44–52 (50)	—	64–88 (76)	—	—
% Genital primordium/total length	62–67 (65 ± 1.37)	—	57–62 (61 ± 1)	—	—	—
Anus§	258–318 (280.5 ± 15.12)	—	287–322 (305 ± 9)	41–51** (46)	—	—
Tail length	19–39 (28.5 ± 4.74)	27–32 (30)	55–64 (59 ± 3)	17–24 (20.5)	—	—
Tail extension (spike)	15–20 (18 ± 1.43)	11–16 (14)	26–32 (29 ± 2)	—	—	—

* Present study, L1 recovered from moose feces, heat-killed and fixed in 70% ethanol, and measured at ×1,000 magnification.
† Pillmore (1956) in snails, bighorn sheep, lambs, and deer may not have included *O. macrotis*.
‡ *Protostrongylus stilesi*, L1 recovered from Dall's sheep feces, heat-killed in water, and measured at ×400 magnification (Kutz et al., 2001).
§ *Protostrongylus rushi*, in bighorn sheep; no unequivocal data have been published. Most infected animals had concurrent infections with *P. stilesi*, hampering isolation of pure material for descriptions of L1 for either species; **measurement of the anus from the tip of the tail.
‖ *Protostrongylus frosti* in bighorn Honess (1942). Morphometrical data were not presented in the original description or subsequently.
Protostrongylus coburni in white-tailed deer. Dikmans (1935) provided a poor description of the L1 and figures depicted a dorsal-spined larvae, these which could have belonged to *Varestrongylus alpenae*, described in same paper, and/or *Parelaphostrongylus tenuis*, which had not been described at that that time. Also, Carreno and Hoberg (1999) stated that after assessing the type series of *P. coburni*, these could not be distinguished from *Protostrongylus boughtoni*.
¶ Distance from anterior end.
†† Dash = No data available.

Our data confirm the placement of *O. macrotis* within the subfamily Protostrongylinae, supported by both morphological and molecular evidence. The spiked-tail among species within Protostrongylinae is considered a synapomorphic character (Carreno and Hoberg, 1999). In addition, our phylogenetic analyses encompassing three different markers, including nuclear ribosomal and mitochondrial DNA, corroborate previous findings, clustering *O. macrotis* within a single clade containing species of *Protostrongylus* (Lesage et al., 2014; Kuchboev et al., 2015). These sequence-based analyses contrast with Carreno and Hoberg (1999), who regarded *Orthostrongylus* as the putative sister of *Protostrongylus* or as the sister of a paraphyletic *Protostrongylus* with *Spiculocaulus*.

Current levels of taxon sampling within the speciose genus *Protostrongylus* remain insufficient for robust conclusions about taxonomic identity. Our analyses appear to support additional partitions for genera within the Protostrongylinae (Fig. 2A), which would require nomenclatural considerations. The phylogenetic placement of *Orthostrongylus* remains equivocal, and comprehensive revision of *Protostrongylus* remains necessary, ideally based on integrated classical and molecular approaches. Many challenges hamper a complete collection of adult stages across an array of ungulates and lagomorphs, including the inherent biodiversity of the genus and varied geographic distributions that encompass remote regions and hosts of varied conservation status (Verocai et al., 2014). Collection of L1s from fecal samples, however, may rely solely on non-invasive methods and can be used for integrated morphological and molecular characterization of species (e.g., Jenkins et al., 2005; Kutz et al., 2007; Verocai et al., 2020).

The lack of morphological data for protostrongylid species, associated with common and broadly distributed North American ungulate hosts, including *P. rushi* and *P. coburni*, may highlight the lack of support or interest for research on the genus (Table I; Dikmans, 1935; Kutz et al., 2001). If possible, the acquisition of additional genetic information among Protostrongylinae should target multiple markers, mitogenomes, or whole-genome sequences. To date, only the mitogenome of *P. rufescens* is available (Jabbar et al., 2013). There is a necessity for extensive and intensive sampling as a basis to establish genetic and taxonomic diversity, and limits for nominal species within Protostrongylinae, and to provide snapshots of historical associations, distribution, and host range across this fauna (e.g., Cook et al., 2017).

The current known range of *O. macrotis* appears to be limited to western North America under prevailing environmental conditions, possibly concordant with the distribution of mule deer and pronghorn. Occurrence in moose and wapiti appears restricted to areas of sympatry with *Odocoileus* and *Antilocapra* (Landram and Honess, 1955; Honess and Winter, 1956; Samuel et al., 1976), consistent with host colonization in the context of ecological fitting in sloppy fitness space (Agosta et al., 2010). The ongoing northward range expansion of mule deer may bring this parasite into sympatry with potentially susceptible ungulates in subarctic and arctic environments (de Vos and McKinney, 2007; Wilson, 2009). As a multi-host lungworm, there is a capacity and potential for it to colonize other ungulates such as caribou (*Rangifer*

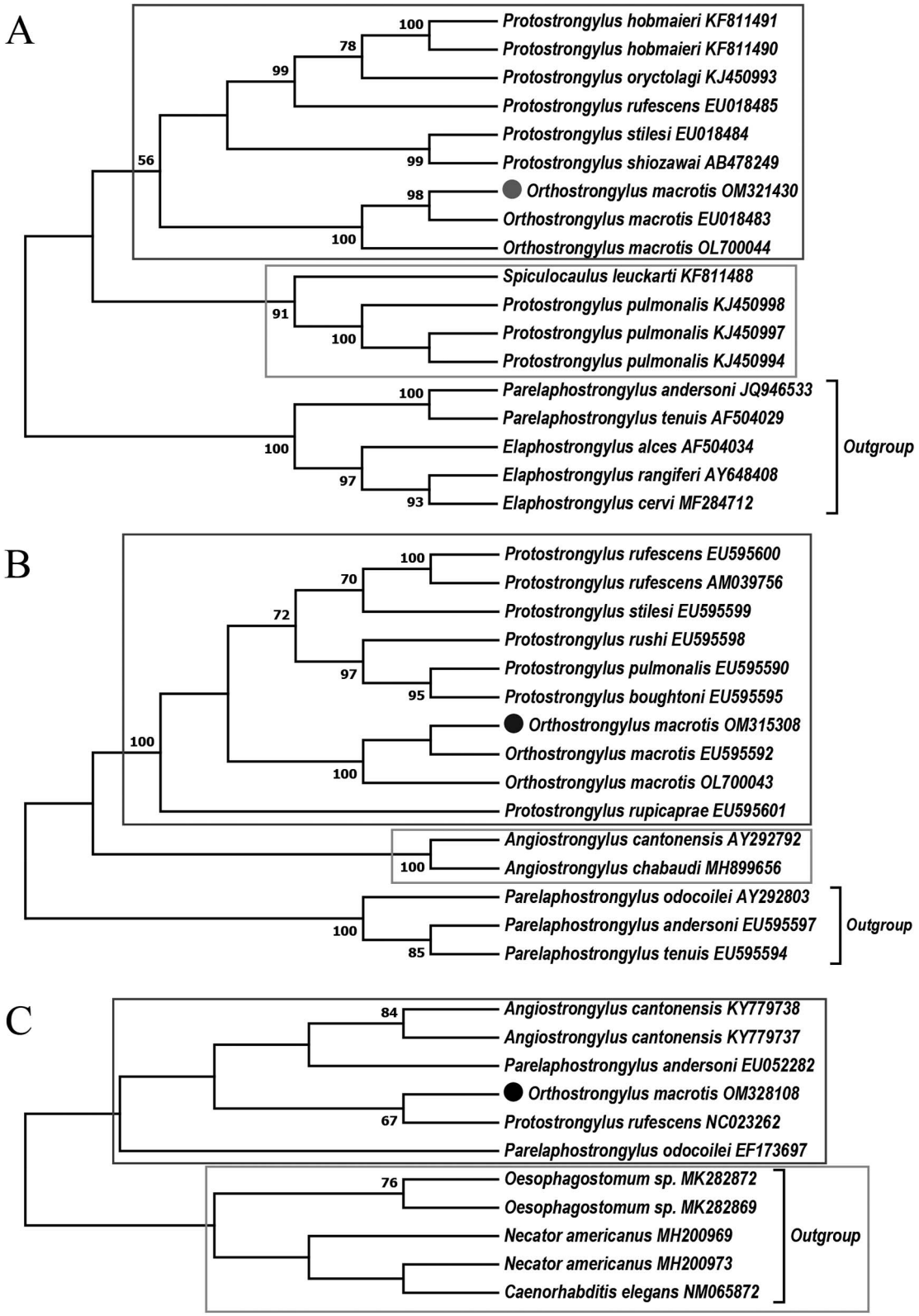

Figure 2. Phylogenetic trees for species identification of *Orthostrongylus macrotis* isolated from feces of male moose (*Alces americanus andersoni*) from Alberta, Canada. (**A**) Phylogenetic tree of the ribosomal Internal Transcribed Spacer subunit 2 (*ITS-2*) gene. (**B**) Phylogenetic tree of the ribosomal Large Subunit (*28S*) gene; (**C**) Phylogenetic tree of the mitochondrial cytochrome *c* oxidase subunit 1 (*COI*) gene. Each panel shows the result of a Maximum Likelihood (ML) method with 1,000 bootstrap replicates; significant bootstrap support values (≥50%) are shown next to the branches.

tarandus), as exemplified by the caribou lungworm, *Varestrongylus eleguneniensis*, that also can establish infections among muskoxen (*Ovibos moschatus*) and moose in areas of sympatry and the context of host movement and changing ecological opportunity (Kutz et al., 2012, 2014; Verocai et al., 2014; Hoberg and Brooks, 2015; Brooks et al., 2019; Kafle et al., 2020). Overall, the literature on *O. macrotis* is sparse and, consequently, its ecology, pathology, and impacts on hosts are unknown.

Most recently, evidence held in GenBank of a genetically similar protostrongyline in a reindeer from the Taimyr Peninsula, Russia, may have brought more complexity to the biogeography of the genus *Orthostrongylus*, and possibly of *O.* cf. *macrotis* (GenBank record attributed to O. A. Loginova and S. E. Spiridonov). This lineage or population may represent a sister species of *O. macrotis* consistent with a historical link between Eurasia and North America during the Pleistocene (e.g., Hoberg et al., 2012). Alternatively, demonstrated is the potential that this occurrence is attributed to anthropogenically driven introduction and colonization, as has been recognized for other nematodes in Eurasian ungulates at high latitudes (e.g., Laaksonen et al., 2015); intensive and extensive sampling among ungulate hosts across the Holarctic are required to evaluate these hypotheses. This recent data have shown that the Russian isolate, compared to existing isolates of *O. macrotis* from North America, demonstrated minimal genetic distance, ranging from 98.9 to 99.5% for *ITS-2* and *28S* genes, respectively. It is not clear if such similarity reflects shallow temporal divergence and relatively recent speciation events against a backdrop of considerable climate and environmental perturbation and isolation during the Pleistocene (Aleuy and Kutz, 2020; see also Asmundsson et al., 2008; Hoberg et al., 2017). Unequivocal confirmation of species identity based on stronger comparative morphological, molecular sequence, and genomic data for larval and adult specimens remains required along with archival deposition of adult and larval specimens (Brooks et al., 2014; Colella et al., 2021).

We explored the morphological basis for identification of L1 attributed to *O. macrotis* in comparison with the few species among *Protostrongylus* and the Protostrongylinae previously characterized from North America. Molecular analyses clearly show a close but unresolved relationship between the two main genera *Protostrongylus* and *Orthostrongylus*. As *O. macrotis* is consistently located within the *Protostrongylus*, this genus may be paraphyletic, as already assumed by Carreno and Hoberg (1999). Biodiversity, biogeography, and host range must be reassessed through integrated classical and molecular approaches. The detailed description of L1 for *O. macrotis* and nascent multilocus phylogenetic assessment may be relevant for a future revision of the genus and differentiation among protostrongylids, with characteristic spike-tails, which infect wild and domestic ungulates from North America and the Holarctic.

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CHAPTER VI

Conclusions

Even though there are no recent updates on dichotomous keys of protostrongylids, we were able to morphologically identify, with a good level of certainty, all the collected parasites. The morphological identification was supported and corroborated by molecular analyzes which confirmed its accuracy and usefulness.

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

The high prevalence (100%) of *Protostrongylus oryctolagi* can be due to the high density of host population together with intermediate hosts availability and a suitable habitat in which larvae, protected by the intermediate hosts, are able to complete their cycle and persist (Guarniero *et al*., 2022).

In support of this hypothesis, several species of snails have been identified as possible intermediate hosts and in particular, the species belonging to the genus *Cernuella* are known to be present in Pianosa (Manganelli *et al*., 2014; Lesage *et al*., 2014).

Moreover, the presence of this single helminth species is supported by its geographic isolation revealing how some environmental factors (i.e., temperature, altitude, seasonal dynamic) influence not only abundance and prevalence but community composition, reinforcing the hypothesis of their stochastically anthropogenic introduction (Bordes and Morand, 2011; Loker and Hofkin, 2015).

Unequivocal species identification based on stronger morphological and molecular data (e.g., the use of new and/or more species-specific genetic markers and genomes) together with the acquisition of genetic population structure and new high-throughput sequencing data, represent a chance to throw light on the history of species belonging to the Protostrongylinae family. To date, only the mitogenome of *P. rufescens* is available (Jabbar *et al*., 2013).

Neglecting parasitological analyses can be extremely disruptive to captive breeding efforts which constantly aim to create viable populations for the conservation of vulnerable and declining species.

The finding that all the sampled hares from Pianosa were proved to be *L. e. meridiei,* together with the identification of a single parasitic species could suggest that, whether not considering some variables such as a randomly sampling of individuals of a single species, both in restocking hares and in those analyzed to evaluate their parasitic fauna, hosts and parasites could have been influenced by population sizes and densities, founder effect, generation time as well by parasites transmission dynamics. Thus, resulting in the creation of a stable and long-lived community which, to date, has not experienced modifications, not even genetic, such as to differentiate them from continental populations.

In particular, natural populations in conditions of geographical isolation offer a chance to deepen the knowledge on how a particular ecosystems works and, as for example in this situation, even on how hosts and parasites coevolve along time. A scientist who recognized the importance of geographical isolated places was Charles Darwin, who observed in his diary *"[…] The zoology of the archipelagos will be worth examining. […]*". Two chapters of "The Origin of Species" were devoted to geographic distribution.

Insular isolation is, in fact, important ecologically because it allows us to be virtually certain that an organism encountered on an island is a true nesiote. Consequently, problems in community structure and function, such as the distribution of individuals into species or the trophic relationships among populations, are more readily attacked in an island setting; any organism found there is assuredly a member of the biotic community (Simberloff, 1974).

For this purpose, more insight studies on this category of endoparasites together with the host-parasite relationships, represent a first step along future path for a better management of wild, semi-domesticated and/or captive breeding populations.

Furthermore, the highlighted inconsistencies brought to light about the taxonomic classification of *O. macrotis*, unveil the necessity for extensive and intensive sampling as a basis to establish genetic and taxonomic diversity, and to provide picture of historical associations, distribution, and host range mainly across wild populations (e.g., Cook *et al*., 2017).

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