

Coprological survey of protostrongylid infections in antelopes from Souss-Massa National Park (Morocco)

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Summary

Protostrongylids, small nematode lungworms, are an integral part of the wild ruminant helminth community, which can damage animals' health when they are held in captivity or semi-captive conditions. The Sahelo-Saharan antelope species dorcas gazelle (*Gazella dorcas*), the scimitar-horned oryx (*Oryx dammah*), and the addax (*Addax nasomacculatus*), reintroduced to Souss-Massa National Park in Morocco, could be host to many species of Protostrongylids. This study was conducted from January to July 2015 to identify infecting parasite species, and determine their prevalence and abundance in all three antelope species. A total of 180 individual fecal samples were collected, morphologically examined by the Baermann technique, and molecularly identified by PCR amplification and sequencing of the second internal transcribed spacer region of the rDNA (ITS-2).

Two parasite species were found in the three antelope populations: *Muellerius capillaris* and *Neostromylus linearis*. The prevalence scores recorded for *M. capillaris* were 98.40 % in the addax, 96.70 % in dorcas gazelle, and 28.40 % in the oryx. The prevalence rates of *N. linearis* were 60 % in the addax, 23.40 % in dorcas gazelle, and 90 % in the oryx. Excreted larvae were quantified by LPG (larvae per gram) counting: for *M. capillaris*, the LPG mean values were 92.94 in the addax, 133.09 in dorcas gazelle, and 1.48 in the oryx; and for *N. linearis*, the LPG mean values were 6.02 in the addax, 1.37 in dorcas gazelle, and 32.81 in the oryx. These findings indicate that the three species of antelopes are infected with *Muellerius capillaris* and *Neostromylus linearis* to varying degrees in intensity and prevalence.

Keywords: *Muellerius capillaris*; *Neostromylus linearis*, prevalence; LPG; threatened antelopes; Souss-Massa National Park

Introduction

Small lungworms (Nematoda: Protostrongylidae) are nematode parasites of a wide range of domestic and wild mammals. Two types of protostrongylids can be distinguished: the meningeal protostrongylids, which parasitize the central nervous system of multiple cervid species (Samuel *et al.*, 2001), and the pulmonary protostrongylids, which parasitize the lower respiratory tract of

many other ruminant and lagomorph species (Taylor *et al.*, 2016). Pulmonary protostrongylid nematodes colonize the bronchi, bronchioles, and alveoli of their hosts. The adult parasite lays eggs in lung tissue, which will hatch *in situ*, producing larvae (first stage L1) that are coughed up, swallowed, and passed in the feces. Once in the environment, the L1 larvae infect terrestrial mollusks (snails and slugs), as intermediate hosts, in which they develop from L1 stage to L3 stage. The L3 larva then infect animals that

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ingest their intermediate hosts, and reach the lungs through the vascular system (Anderson, 2000).

These nematodes are not only the primary infectious agents of the classical verminous pneumonia, but they also make damaged lung tissue susceptible to secondary bacterial infections (Kaufmann, 2013). Symptoms of infection in wildlife are not visibly obvious, and differ from one host to another, and the health impacts vary from sporadic observance upon necropsy (Kabakci *et al.*, 2007; Panayotova-Pencheva & Alexandrov, 2010) to massive associated mortality (Forrester, 1971; Demartini & Davies, 1977).

Prevalent worldwide, protostrongylids are more widely studied in domestic ruminants (Kuchboev *et al.*, 2017; Ahmadi *et al.*, 2018; De Macedo *et al.*, 2020) due to our economic interest in them, than in wild fauna. In the Mediterranean basin, several studies have been conducted in free-ranging wildlife, especially from the north shore: in wild caprinae from France, Spain, Italy and Portugal (Nocture *et al.*, 1998; Acevedo *et al.*, 2005; Cassini *et al.*, 2015); in lagomorphs from France, Italy and Bulgaria (Lesage *et al.*, 2014; Sergi *et al.*, 2018; Panayotova-Pencheva *et al.*, 2019), and in different species of ungulates from Iberian peninsula (Morrondo *et al.*, 2017; Figueiredo *et al.*, 2020). By contrast, no studies of protostrongylids in wild mammals have ever been conducted on the Mediterranean south shore (North Africa), particularly in antelopes.

This study was performed to fill this gap by exploring protostrongylid infections in three endangered antelope species, which previously inhabited the desert zones of Morocco and North Africa (Loggers *et al.*, 1992). Their reintroduction happened in 1995 at Souss-Massa National Park (SMNP) in Agadir (south of Morocco). The three species are *Gazella dorcas* (Linnaeus, 1788), *Oryx dammah*, (Cretzschmar, 1826) and *Addax nasomaculatus* (De Blainville, 1816). The oryx and addax were introduced from different European zoos, while dorcas gazelle was repopulated from a native wild population (Müller & Engel, 2004). These three species appear on the Red list of threatened species of the International Union for Conservation of Nature (IUCN). The dorcas gazelle is listed in the «vulnerable» category (IUCN, 2017), the addax in the «critically endangered» category (IUCN, 2016a), and the Scimitar-horned oryx in the «extinct in the wild» category (IUCN, 2016b).

Our objective was to identify the protostrongylid parasite species

found in these endangered antelope species, and measure the intensity of infection in each host species. We opted for non-invasive methods to complete this survey. First, we used fecal examination for qualitative (larval morphologic characterization) and quantitative (larval counting) analyses. Second, we used molecular tools (sequencing the second internal transcribed spacer (ITS-2) of the nuclear ribosomal DNA (rDNA)) to identify species of isolated nematode larvae using universal primers (Gasser *et al.*, 1993).

Materials and Methods

Study area and animals

Souss-Massa national park (SMNP) is located near the city of Agadir (south of Morocco), extending from Oued Souss estuary in the north, to the Massa estuary in the south. Its center is at 9°40'W 30°5'N. Its climate is semi-arid Mediterranean, with an Oceanic influence. The park is home to a large variety of endemic flora (e.g. *Argania spinosa*) and fauna (e.g. *Geronticus eremita*).

To avoid hybridization related problems between addax and oryx, the animals are isolated from each other in two separate reserves within the SMNP. The addax and part of the dorcas gazelle population reside in the first reserve, called «Rokein» while the oryx and the rest of the dorcas gazelle population reside in the second reserve named «Arrouais». The estimated population sizes are approximately 230 oryx, 440 addax, and 850 dorcas gazelles.

Sampling methodology

Individual samples of fresh fecal pellets of correspondent herds were randomly collected from the ground during morning hours, according to their availability, from different sites within the park, and regardless of the sex and age of animals. A total of 180 samples, representing all three species of antelope, were collected between January and July of 2015. All samples were labeled, transported immediately to the laboratory, and refrigerated at 4°C until used.

Laboratory analyses

Larvae recovery and counting

First stage larvae (L1) were extracted from fecal pellets and counted (larvae per gram of feces: LPG) using the modified Baermann technique described by Cassini *et al.* (2015).

Table 1. Prevalence rates and LPG values extracted from feces of each species of Protostrongylids for the three populations of antelopes.

	Addax (n = 60)		Gazelle (n = 60)		Oryx (n = 60)	
	<i>M. capillaris</i>	<i>N. linearis</i>	<i>M. capillaris</i>	<i>N. linearis</i>	<i>M. capillaris</i>	<i>N. linearis</i>
LPG Mean	92.94	6.02	133.09	1.37	1.48	32.81
LPG Max	542	40	951.7	20	20	838.4
LPG Min	0	0	0	0	0	0
Standard error	13.42	1.70	23.09	0.44	0.50	13.99
Prevalence %	98.40	60.00	96.70	23.40	28.40	90.00

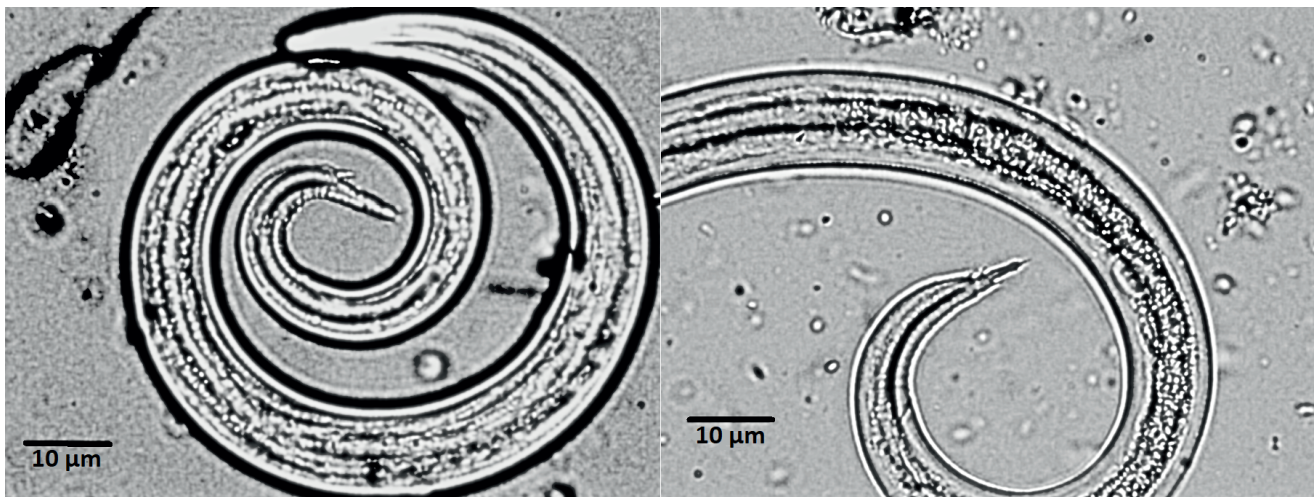


Fig. 1. Protostrongylid L.1 larvae patterns recovered from antelopes, *Muellerius capillaris* (left) and *Neostrongylus linearis* (right), showing the morphological characteristics of the tail.

Larvae were recognized as protostrongylid larvae by morphological characteristics, particularly of their tail (Boev, 1975). In general, genera are easily identified (and some genera present only one species). Hence, one of each morphologically unique larva was isolated from each host under inverted microscope, and placed in 20 µl of water in a 0.5 mL tube, and preserved at -20°C for molecular analysis. For phylogenetic comparisons, L1 larvae from Moroccan domestic sheep were also isolated.

DNA extraction

DNA was extracted according to the tissue protocol using a QIAGEN QIAamp DNA Mini Kit (QIAGEN Lake Constance GmbH, Germany) with a slight modification in the tissue digestion step: 180 µl of ATL buffer and 20 µl of Proteinase K were added to the 0.5 ml tube containing the larva, vortexed and centrifuged for a few seconds, then the contents were transferred to a 2 ml tube containing 0.5 mm Zirconia/Silica beads (BioSpec®, BioSpec Products, Inc., USA) and incubated in a thermal shaker (CAT®-H26) for

1 h at 56°C / 900 rpm. Finally, 200 µl of the digestion product was collected for DNA isolation by the QIAGEN protocol.

PCR amplification

PCR assays targeted the internal transcribed spacer (ITS-2) regions of the nematode ribosomal DNA. Universal primers described by Gasser *et al.* (1993) (forward primer NC1: 5'ACGTCTGGTTCAGGGTTGTT; reverse primer NC2: 5'-TTAGTTTCTTTTCCTCC GCT-3') were used for amplification.

PCR amplification was performed using the AgPath-ID™ Master Mix kit (Applied Biosystems, Foster City, CA, USA) in a total volume of 50 µl per reaction. Each reaction contained 10 µl of DNA extract, 2 µl of each 10 µM primer, 2 µl of 25X RT-PCR Enzyme Mix, 25 µl of 2X RT-PCR Buffer and 9 µl of water. PCR reactions were carried out in a Bio-Rad iCycler® Thermal cycler with initial denaturation at 94°C for 10 min, followed by 40 cycles (45 seconds each) of denaturation at 94°C, annealing at 55°C and extension at 72°C, with a final extension at 72°C for 5 min. PCR amplicons

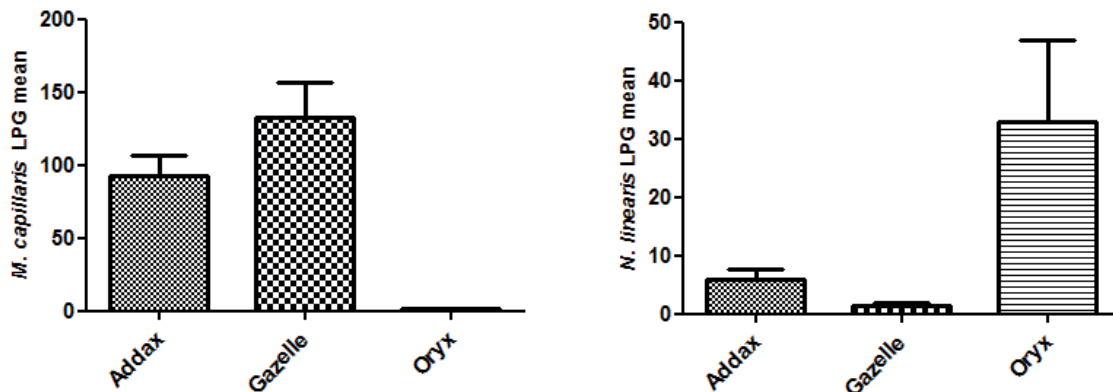


Fig. 2. Comparative LPG means in the all three antelopes for *Muellerius capillaris* (left) and *Neostrongylus linearis* (right).

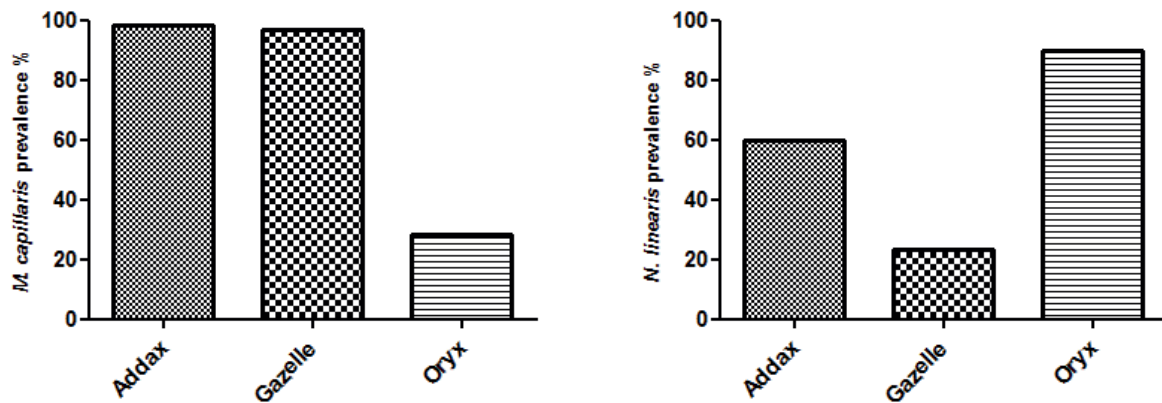


Fig. 3. Comparative prevalence rates across the three antelopes for *Muellerius capillaris* (left) and *Neostromylyus linearis* (right).

were visualized by electrophoresis in 2 % agarose gel. Positive samples were identified by the presence of a 425 bp band.

DNA sequencing and phylogenetic analysis

PCR products were submitted to a sequencing service provider (BIO BASIC®, Markham ON, Canada) for purification and Sanger dideoxy sequencing using primers NC1 and NC2. Electropherograms were examined by Sequence Scanner Software v1.0 (Applied Biosystems). The GenBank database was searched for sequences that matched the sequencing results using the BLAST algorithm hosted by the national center for biotechnology information (NCBI) network server (Johnson *et al.*, 2008; Benson *et al.*,

2014). Sequence alignments were performed using *BioEdit* v7.2.6 software (Hall, 1999), and phylogenetic analyses by MEGA v7.0 software (Kumar *et al.*, 2016). Phylogenetic trees were constructed by the neighbor joining method after 1000 bootstraps (Saitou & Nei, 1987). Eleven sequences were deposited in the Genbank database (Accession numbers from MN543050 to MN543060).

Statistical analysis

Prevalence was expressed as percentages, and LPG values as means \pm standard deviations. Differences in prevalence were analyzed using the χ^2 test (95 % confidence interval), and LPG values among the three populations of antelopes were compared with one-way analysis of variance (ANOVA) and the Newman-Keuls

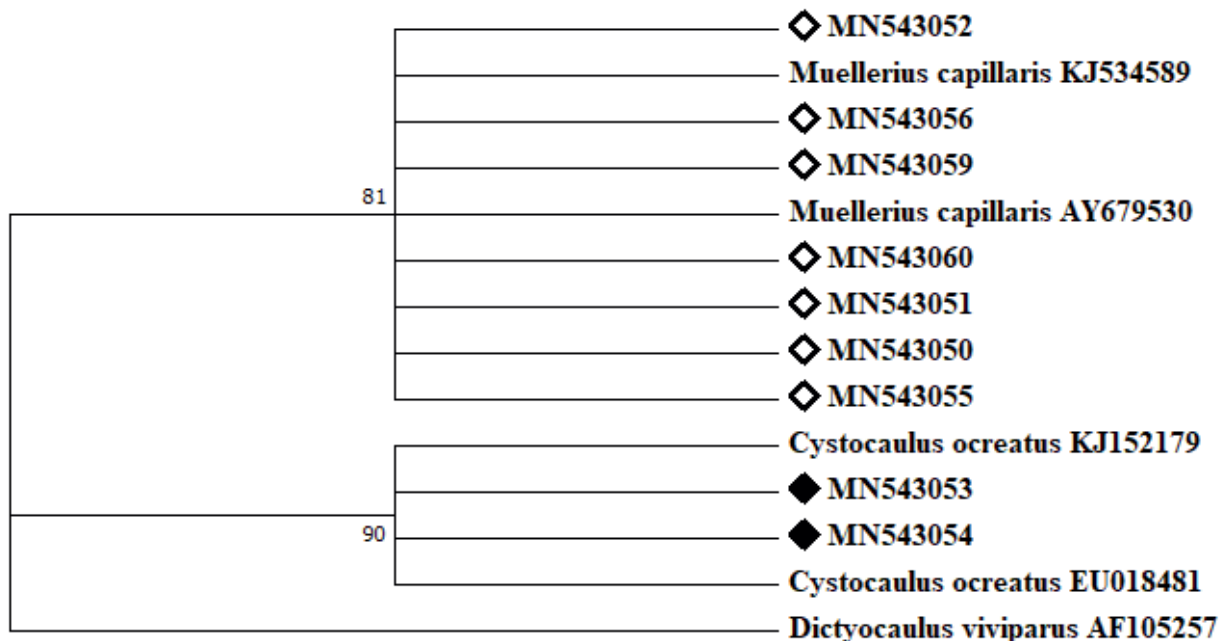


Fig. 4. The condensed phylogenetic relationships of Protostrongylids based on ITS-2 sequences of the rDNA, using the Neighbor-Joining method with the Tamura 3-parameter method. The percentages of replicate trees in the bootstrap test (1000 replicates) are shown next to the branches. The analysis involved 14 rDNA ITS-2 nucleotide sequences (5 from antelopes, 4 from local sheep and 5 from Genbank). Evolutionary analyses were conducted in MEGA7.

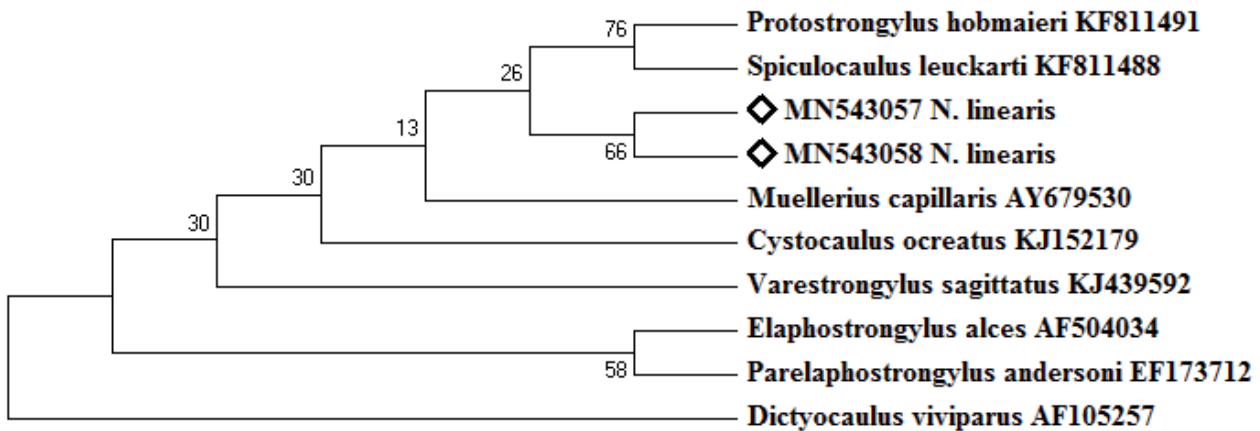


Fig. 5. The phylogenetic relationships showing topology of Protostrongylids using the Neighbor-Joining method with the Tamura 3-parameter method with the bootstrap test (1000 replicates) shown next to the branches. The analysis involved 10 rDNA ITS-2 nucleotide sequences (2 from antelopes and 8 from Genbank). Evolutionary analyses were conducted in MEGA7.

multiple comparison test, with P-values of <0.05 indicating significance (GraphPad PRISM® v5.00 software, USA).

Ethical Approval

Approval of animal care and use committee was not required; this study did not conduct any animal experiments.

Results

Morphological characterization of recovered larvae

Two distinguishable morphological patterns of L1 larvae were isolated from all three antelope species. The two morphological forms of the isolated larvae match with those of *Muellerius capillaris* and *Neostromylylus linearis*. *Muellerius capillaris* is a dorsal spiny-tailed larva with an average length of 310 µm; however *Neostromylylus linearis* is also a dorsal spiny-tailed larva, but with two additional terminal spines, and measures about 350 µm long (Fig. 1).

Prevalence and LPG values

Parasite larvae counts (LPG), and parasite prevalence values are shown in Table 1. The results of ANOVA show statistically significant differences in LPG values for *Muellerius capillaris* (F 19.10; p<0.05) among the three antelopes populations. The same is true for *Neostromylylus linearis* (F 4.339; p<0.05).

LPG values for each parasite in all three antelope populations are compared in Figure 2. The highest LPG for *Muellerius capillaris* was in the dorcas gazelles, while the lowest was in oryx. These results are reversed for *Neostromylylus linearis*: the highest LPG was recorded in oryx, and the lowest LPG was in dorcas gazelles. The χ^2 test showed that *Muellerius capillaris* is significantly more

prevalent in the addax and dorcas gazelle populations compared to the oryx population. *Neostromylylus linearis* is significantly more prevalent in the oryx population than the addax and dorcas gazelle populations. Its prevalence is also higher in the addax population than the dorcas gazelle population (Fig. 3).

Molecular identification and phylogenetic analyses

Eleven sequences were subjected to similarity searches using the BLAST algorithm of NCBI. The sequences isolated from domestic sheep were confirmed to be *Muellerius capillaris* with accession numbers MN543059 and MN543060, and *Cystocaulus ocreatus* with accession numbers MN543053 and MN543054. Homology between sequences is given in the phylogenetic tree in Figure 4. Phylogenetic analysis of the *Muellerius capillaris* sequences, isolated from Dorcas gazelle (MN543055, MN543056) and Addax (MN543050, MN543051, MN543052) revealed that it clusters well with the *Muellerius capillaris* clade including the sequences obtained from local domestic sheep, the reference sequences isolated from muskox (*Ovibos moschatus*) in Norway (KJ534589), and the reference sequence isolated from goat (*Capra hircus*) in Canada (AY679530). Similarities between isolates and reference sequences are represented in the phylogenetic tree in Figure 4. Phylogenetic analysis of *Neostromylylus linearis* sequences (MN543057, MN543058) showed that it does not cluster with the *Muellerius capillaris* or *Cystocaulus ocreatus* clades. This protostrongylid is phylogenetically more closely related to other genera, especially *Protostrongylus hobmaieri* and *Spiculocaulus leuckarti*, which belong to the Protostrongilinae sub-family. Figure 5 provides information on genetic topology and distances between these isolates.

Discussion

Our morphological and molecular studies revealed that these three antelope species are obviously infected by two species of protostrongylids: *Muellerius capillaris* and a *Neostrongylus linearis*.

Prevalence and intensity of infection vary among antelope species as shown previously. Compared to other studies conducted under similar conditions, such as Cassini *et al.* (2015), the prevalence and LPG values of *Muellerius capillaris* in dorcas gazelle seem to be closer to those found in ibex (*Capra ibex*). By contrast, LPG values for *M. capillaris* in addax and oryx were very low even though its prevalence is high in addax. However, with comparison to the same study, the prevalence recorded for *Neostrongylus linearis* in the three antelopes was higher than that recorded in the ibex, but the LPG amounts registered in antelopes were lower than that noticed in ibex, excepted for the oryx that recorded a higher LPG amount compared to the ibex.

This variation in excreted larval load (LPG) depends on many factors. The fecal larval output correlates positively with parasite burden, and is linked to intermediate host (terrestrial mollusks) abundance, which is influenced by climatic parameters (moisture and temperature). Many species of terrestrial gastropods which could be implicated in the ecology of protostrongylids have been identified in the geographical area of Souss-Massa estuaries. These include *Theba subdentata meridionalis*, *Otala lactea*, *Rumina decolata*, *Rumina saharica*, *Ferussacia moreleti*, *Cochlicella acuta* and *Cochlicella Barbara* (Irikov and Gerdzhikov, 2013). In addition, the specificity of parasites to their hosts is variable and may explain the high interspecific variation in infection intensity. Some stress factors could also impact larval output amounts. These may be intrinsic (physiological state: e.g. pregnancy) or environment related conditions (Cabaret *et al.*, 1980; Díez-Baños *et al.*, 1994). Wildlife health management doesn't consider the health of individuals only, as in domestic fauna. It is based more on monitoring population health (Wobeser, 2007) or ecosystem health, and uses a multidisciplinary approach (Aguirre *et al.*, 2002). Parasites have been shown to influence the dynamics of their host populations by reducing fecundity and survival (Tompkins & Begon, 1999). Under natural conditions, when animal populations are left to manage on their own, a balance is established between host populations and their specific parasites (Jacobs *et al.*, 2015). In cases of endangered species that we have to preserve under sequestered conditions in natural reserves and parks, a dashboard of follow-up indicators including parasitic infections should be put in place (Heard *et al.*, 2013; Stringer & Linklater, 2014).

For wildlife conservation purposes, we have resorted to introduction, reintroduction, and translocation programs, which will impact the ecology and biodiversity of the parasites they carry (Moir *et al.*, 2012). Dissemination of introduced parasites in new ecosystems could become a hazard to endemic potential host species (Malan *et al.*, 1997; Leighton, 2002).

The results of this current study have become part of a dashboard

of health indicators that will serve wildlife management programs in the Souss-Massa National Park for routine monitoring, or for further animal translocation operations. These studies should be extended to examinations of adult parasites in eventual cadavers to characterize adult nematodes in each antelope host, and to the infective larvae in the intermediate hosts to identify snail species implicated in the life cycle of each protostrongylid species.

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Conflict of Interests

Authors state no conflict of interests.

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