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Parasite diversity in grey wolves (*Canis lupus*) from Tuscany, central Italy: a copromicroscopical investigation

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A B S T R A C T

Parasite monitoring is considered an important issue for wildlife conservation as well as for veterinary and public health. In Italy, data on endoparasites of the grey wolf (*Canis lupus*) are still limited. A copro-microscopical study was performed to investigate endoparasites of grey wolf communities in three Apennine natural reserves and a hilly area in northern Tuscany, central Italy. A total of 66 fresh faecal samples were collected and examined using the Mini-FLOTAC technique with a high-density flotation solution. Apparent prevalence and 95 % confidence intervals (CI) were estimated for each parasite taxon, while true parasite prevalence rates were assessed for individual wolves identified after molecular analyses in one of the three natural areas (Apuan Alps Regional Park, AARP). Overall, a high prevalence of endoparasites was estimated (92.4 %, 95 % CI 83.2–97.5), and most samples were found positive for respiratory capillariids (81.8 %), i.e., *Eucoleus boehmi* (66.7 %) and *Eucoleus aerophilus* (31.8 %), *Sarcocystis* spp. (36.4 %) and hookworms (21.2 %). *Physaloptera* spp. (7.6 %), *Toxocara canis* (1.5 %), *Spirocerca lupi* (1.5 %) *Crenosoma vulpis* (1.5 %), *Angiostrongylus vasorum* (1.5 %), *Opistorchis felineus* (3 %), *Alaria alata* (1.5 %), Taeniids (12.1 %), and *Cystoisospora* spp. (6.1 %) were also identified. *Dicrocoelium dendriticum* eggs and *Demodex* spp. mites were detected in few samples. No significant differences emerged between faecal and population prevalence for any of the parasite species identified in AARP. Findings from this study add new information on grey wolf endoparasite infections in Italy and confirm the high prevalence of respiratory capillariids circulating among wild canids in Europe. Our results highlight the important role grey wolves may play in the transmission of these capillariid species between wild and domestic canids as well as of potentially zoonotic parasites in examined areas. Moreover, some endoparasites identified in this study may negatively affect the health of infected wolves.

1. Introduction

In Italy, up to the 19th century, the grey wolf (*Canis lupus* Linnaeus, 1758) was common and widely distributed across the peninsula (Coppola et al., 2022). However, as a result of deforestation, human persecution, and environmental changes, by the 1970s only about 100

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wolves were left in Italy, located mainly in the central-southern Apennines (Zimen and Boitani, 1975). From the end of the 1970s, Italian wolf populations have increased to approximately 3300 individuals, expanding mainly from the central Apennines to recolonize the northern Apennines and western Italian Alps, in wild as well as in rural anthropized environments (Fabbri et al., 2007; Macchioni et al., 2021; Mattioli et al., 2018; La Morgia et al., 2022). Since 1992, the wolf has been a protected species in Europe (Council Directive 92/43/EEC on the Conservation of Natural Habitats and of Wild Fauna and Flora).

Endoparasites of the grey wolf include species that are transmitted between canids and as a result of predator-prey interactions (Stronen et al., 2021). Interestingly, wolves with a more diversified diet may show a lower number of parasite taxa characterized by an indirect life cycle (Molnar et al., 2019). Knowledge and monitoring of parasitic fauna are crucial for the conservation of wildlife, including the grey wolf. Indeed, parasites may have an impact on this predator and its related prey populations, even influencing predator prey dynamics. Such monitoring can also indicate parasite transmission between domestic and wild canids, as well as between wolf and its prey, both domestic and wild (Molnar et al., 2019; de Macedo et al., 2020; Stronen et al., 2021). This knowledge is important since the recent recolonization of grey wolves may also increase parasite infection risk in prey and in domestic dogs (Lesniak et al., 2018; Molnar et al., 2019).

Parasite monitoring is also essential for veterinary and public health since zoonotic pathogens can spread from wild to domestic canids, as well as from wild canids to domestic livestock and vice versa (Molnar et al., 2019; de Macedo et al., 2020; Stronen et al., 2021). Indeed, several parasites infecting wild and domestic canids may have a zoonotic potential (Otranto et al., 2015; Poglayen et al., 2017; Bezerra-Santos et al., 2022; Ortega et al., 2024). Predation on livestock by wolf populations living in anthropized rural areas of Italy can represent a risk for *Echinococcus granulosus* infection in wolves, due to the high prevalence of cystic echinococcosis in small domestic ruminants (Poglayen et al., 2017; Macchioni et al., 2021).

Geographical factors and local prey populations have been also reported to influence the diversity of parasite species infecting the grey wolf (Lesniak et al., 2017; Molnar et al., 2019). Wolves living in an Italian park were found infected by a higher number of directly transmitted parasites compared to wolves living in parks of other countries (Molnar et al., 2019).

Endoparasites most frequently identified in wolf faecal samples in Italy up to now include nematodes, mainly *Eucoleus boehmi*, *Eucoleus aerophilus,* hookworms and ascarids, taeniid cestodes, and some protozoa, mainly *Sarcocystis* spp., *Giardia duodenalis* and *Cystoisospora* spp. (Mariacher et al., 2015; Molnar et al., 2019; Paoletti et al., 2017; Poglayen et al., 2017; Macchioni et al., 2021; Guadano Procesi et al., 2022; Perrucci et al., 2023).

To date, however, data on gastrointestinal and (cardio)respiratory parasites of wolves in Italy are still limited, especially in northern Apennine areas (Mariacher et al., 2015; Poglayen et al., 2017; Macchioni et al., 2021; Perrucci et al., 2023). Therefore, here we performed a copro-microscopical study to investigate endoparasites of wolf communities in four areas of northern Tuscany, central Italy, including three natural reserve areas of the northern Apennines.

2. Materials and methods

2.1. Study areas and sample collection

This study was carried out in the Apuan Alps Regional Park, the Gherardesca Lake protected area, the Sibolla Lake Nature Reserve, and the municipality of Crespina-Lorenzana in the Provinces of Massa

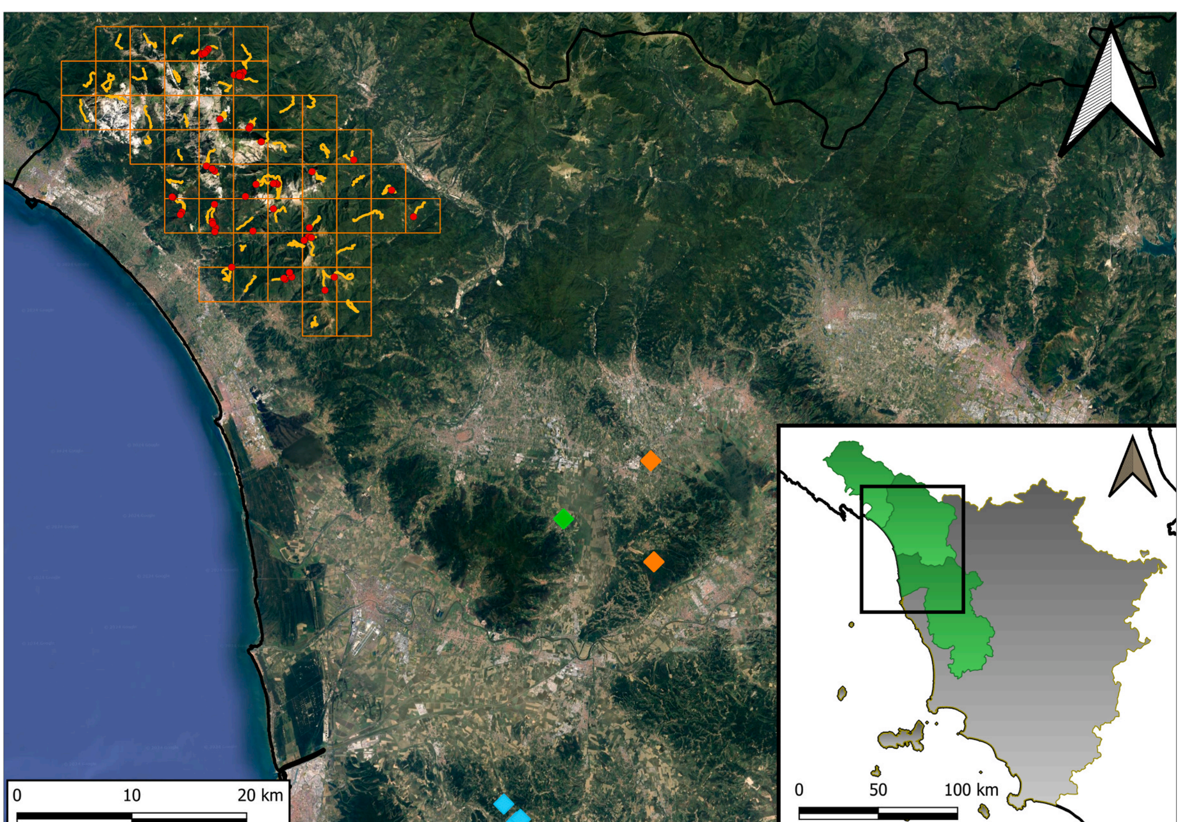

Fig. 1. Sampling areas from northern Tuscany, central Italy, where grey wolf faecal samples were collected between February 2020 and March 2022. The four study areas are Apuan Alps Regional Park (orange grid with fixed routes in yellow and collected scats in red), Gherardesca Lake (green diamond), Sibolla Lake Natural Reserve (orange diamonds), Crespina-Lorenzana (light blue diamonds).

Carrara, Lucca and Pisa, Region of Tuscany (central Italy) (Fig. 1), respectively, where the grey wolf is the only large predator of the carnivore guild (Coppola et al., 2022; Zanni et al., 2023).

The Apuan Alps Regional Park (206 km^2) and surrounding area (314 km^2) (hereafter referred to together as AARP), with an elevational range of 200–1946 m above sea level, is a southward protrusion of the northern Apennines, 5–10 km north of the Ligurian Sea. AARP is characterized by a typical alpine environment and includes several geographical areas belonging to the Provinces of Lucca (Garfagnana and Versilia) and Massa Carrara (https://www.parcapuane.toscana.it), and it is considered one of the rainiest areas in Italy (Agnoletti, 2007). Vegetation varies dramatically from Mediterranean scrub on the coast hills to oak-hornbeam (*Quercus* spp.- *Ostrya carpinifolia*) associations, chestnut (*Castanea sativa*) and beech (*Fagus sylvatica*) woods at higher elevation, ending with montane grasslands or bare cliffs above the tree line. According to the latest wolf howling survey (Fazzi and Lucchesi, 2024) and the criteria established in other Apennine areas to distinguish different packs (Apollonio et al., 2004), the wolf population in this area has been estimated to be at least seven breeding units. The carnivore guild also includes medium sized and small carnivores, such as red fox (*Vulpes vulpes*), European badger (*Meles meles*), stone (*Martes foina*) and pine (*Martes martes*) martens, European polecat (*Mustela putorius*), European wildcat (*Felis silvestris silvestris*) and common weasel (*Mustela nivalis*). Although protected, recreational activities are permitted in the area, whereas the surrounding region is impacted by human activities including marble quarrying and hunting.

The Gherardesca Lake wetland (hereinafter GL) is permanently flooded and covers over 40 ha in the municipality of Capannori, Province of Lucca. This lake basin extends into a wetland nature protected area established along bird migration routes, where hunting is prohibited (https://it.wikipedia.org/wiki/Lago_della_Gherardesca). The lake area is surrounded by green areas with meso-hygrophilous and hygrophilous vegetation, as well as cultivated land composed of forest, wetland, marsh, and reed beds (*Phragmites australis*). The forested area consists of deciduous oak (mainly *Quercus cerris*) with a shrub-rich undergrowth. The carnivore guild includes red fox, stone marten and common weasel. Wolf presence has been recorded in GL; however, no data on population size are available.

The Sibolla Lake Nature Reserve (hereafter SL), consisting of 60 ha of swamp and forest, was established in 1996 in the municipality of Altopascio, Province of Lucca, and was recognized as a Site of Community Interest in 2000 (https://it.wikipedia.org/wiki/Lago_di_Sibolla). In addition to the wolf, red foxes and mustelids (common weasel, European polecat and badger) are present (http://sira.arpat.toscana.it/sira/MedWet/MDW_IT51262301.htm). Again, wolf presence has been recorded, but wolf abundance is unknown.

The municipality of Crespina-Lorenzana (hereinafter CL) is located in the hills about 30 km south-east of Pisa. The territory is characterized by small urban centres interspersed with crops (olives, cereals, and fodder) and extensive or semi-extensive pasture (sheep or cattle), with small wooded areas (Coppola et al., 2022). Wild carnivores living in this area include wolf, red fox, pine and beech marten, and European polecat (Coppola et al., 2022). A single wolf pack is also become established, and has been monitored since 2018 (Coppola et al., 2022).

To systematically collect fresh wolf faecal samples for parasitological examination and individual genotyping, AARP was divided into a grid of 3×3 km squares, with a fixed walking route of at least 2 km (2782.60 $\pm$ 888.56 m, mean $\pm$ SD) in each cell, walked bimonthly in 2020 and quarterly between January 2021 and March 2022. In all remaining sampling areas, fresh samples were collected along fixed pathways between July 2022 and March 2023. The freshness of faecal samples was determined based on external appearance and local weather conditions. The samples collected in AARP were selected from a larger dataset for an additional ongoing project on *Echinococcus* spp. infection in wolves in this area (Cafiero et al., 2025), and stored for 5 days at −80 °C to deactivate any *Echinococcus* spp. eggs (Veit et al., 1995). The samples collected in all other sampling sites were stored at −20 °C until examination.

Since most samples were not individually genotyped, we cannot rule out the possibility that we resampled the same individuals on different days (Marathe et al., 2002; Mech and Boitani, 2003). Therefore, to correctly estimate parasite prevalence, the population size of wolves should also be estimated. However, population size could only be calculated for AARP since this study area was screened for a longer period and more systematically, thus providing an adequate sample size. Therefore, for samples collected in 2021 and 2022 from AARP (43 samples), host cells were collected by swabbing the mucosal surface of fresh faecal pellets in the field using a Genotube (Prionics Lelystad B.V., ThermoFisher, Netherlands) and preserved in safe lock Eppendorf tubes at room temperature as recommended by the manufacturer until molecular analyses for individual genotyping.

2.2. Parasitological analyses

Parasitological analyses were performed at the Parasitology Laboratory of the Department of Veterinary Sciences, University of Pisa. For each faecal sample, 2 g subsamples were examined using the Mini-FLOTAC technique with a detection limit of five protozoan cysts/oocysts/sporocysts or five helminth eggs per gram of faeces, in 18 ml of a high-density flotation solution (zinc sulphate solution, 1.35 specific gravity), for the detection and quantification of parasitic organisms with both low and high specific weight (Maurelli et al., 2014; Bosco et al., 2020; Ianniello et al., 2020). A flotation test (2 g subsamples in 18 ml of the same flotation solution) was also used for microscopical evaluation of morphometrical features of parasitic elements at high magnification.

Detected parasites were microscopically identified to the lowest taxonomic level based on their size, colour, and shape (Foreyt, 2001; Genchi et al., 2010; Taylor et al., 2022). Taeniid eggs were classified to Family level (Thompson, 2017). *Eucoleus boehmi*, *E. aerophilus* and *Trichuris vulpis* eggs were differentiated at high magnification by evaluating the aspect of their contents, the structure of the eggshell surface, and the egg size (Traversa et al., 2011; Di Cesare et al., 2012; Magi et al., 2012; Perrucci et al., 2014; Morelli et al., 2021). Dimensions were measured with a micrometric ocular.

2.3. Individual genotyping

In order to estimate parasitic true prevalence in wolves from AARP, individual genotyping was performed on faeces collected in 2021–2022, following the protocol recommended by the Italian Institute for Environmental Protection and Research (ISPRA), with minor modifications (Caniglia et al., 2014, 2020). Sample processing, DNA extraction and amplification were carried out at the Animal, Environmental and Antique DNA Platform (Conservation Genomics Research Unit), Fondazione E. Mach (FEM), Italy, and all sequencing was performed at the FEM Sequencing and Genotyping Platform. Briefly, DNA extraction from faecal swabs was performed using Mag-Bind Blood & Tissue DNA HDQ 96 kit (Omega Bio-Tek, Inc., Narcross Georgia 30071 USA). A 250–500 bp portion of highly variable WD-Loop mitochondrial gene was amplified for preliminary species discrimination. The *zinc-finger protein* ZFX/ZFY *locus* (Garcia-Muro et al., 1997) that is sited on sexual chromosomes was targeted for sex determination (Lucchini et al., 2002). A set of multilocus microsatellites (CPH5, Cl 09.250, Chroy, FH2088, FH2096, CPH2, CPH4, CPH8, FH2004, C20.253, CPH12, FH2079) (Fredholm and Wintero, 1995; Francisco et al., 1996) was amplified in multiple replicates per locus (four or eight in case of analysis issues) for characterisation of individual genotypes. The individual genotype was built by comparing all the replicates through GIMLET v.1.3.3. software (Valière, 2002) and with the database of wolf genotypes available at FEM.

2.4. Statistical analyses

Apparent prevalence and 95 % confidence intervals (CI) were estimated using R (version 4.4.2) for each parasite taxon, both from the whole dataset and for each study area. To avoid potential bias from individual resampling and from re-infection rate, parasite prevalence in AARP was also assessed for individual wolves identified by STR genotyping. True prevalence was calculated by dividing the number of positive genotypes by the total number of identified individuals (Liccioli et al., 2015). A Fisher exact Chi-square test was used to compare faecal and population prevalence for all parasite taxa, as well as to compare both faecal and true prevalence separately between the two *Eucoleus* species detected.

3. Results

A total of 66 fresh faecal samples of grey wolves were collected and analysed. Fifty-five faeces were collected in AARP, while 11 were collected from the other three study sites, GL, SL, and CL (1, 5 and 5 samples, respectively). The mean prevalence and intensity of parasite environmental stages for each examined area are shown in Table 1, while a list of coinfections in AARP, in Table 2. Individual genotyping was successful for 23 out of 43 faecal samples in AARP and identified 16 individuals. One faecal sample each was used to identify 10 individuals, two faeces each were used to isolate five wolf genotypes, and one was extracted from three faecal samples.

Overall, a high prevalence of endoparasites was found (61/66; 92.4 %, 95 % CI 83.2–97.5), including a high number of coinfections (66.7 %, 95 % CI 54.1–78.2). No significant differences were found between faecal and population prevalence rates for each parasite taxon in AARP (Table 1).

Capillariid eggs were highly prevalent in the wolf faecal samples we collected (54/66, 81.8 %), and both *E. boehmi* (44/66; 66.7 %) and *E. aerophilus* (21/66; 31.8 %) were identified, with more than 20 % coinfections (15/66; 22.73 %) (Table 2; for four samples, capillariid eggs could not be identified to species level). *E. aerophilus* showed a statistically different (p-value <0.01) overall lower prevalence (31.8 %) than *E. boehmi*. The prevalence of these two parasite species was particularly high in AARP, where 76.4 % (42/55) and 29.1 % (16/55) of samples were positive for *E. boehmi* and *E. aerophilus*, respectively (Table 1). Moreover, 95 % CIs for faecal prevalence of these two parasites in AARP did not overlap at all (Fisher exact Chi-square test: p-value $= 1.17 \times 10^{-6}$) (Fig. 2). Although the true prevalence CIs of these two species partially overlapped, the difference remained statistically significant (p-value $= 0.0233$) (Fig. 3).

As for the remining nematodes of the (cardio)respiratory tract identified in this study, first stage larvae of *Crenosoma vulpis* were found in one faecal sample from CL and *Angiostrongylus vasorum* was detected in one sample from SL.

Ancylostomatids, *Ancylostoma caninum* and *Uncinaria stenocephala*, showing an overall prevalence of 21.2 % (14/66), were the most common gastrointestinal nematodes, and were found in three of the four study sites. Each of the three other gastrointestinal taxa were found in a single study site with lower prevalence: *Physaloptera* spp. (7.6 %; 5 samples in AARP), *Toxocara canis* (1.5 %; one sample in SL) and *Spirocerca lupi* (1.5 %; one sample in CL) (Table 1).

Two faecal samples (2/66; 3 %) from AARP were found positive for eggs resembling those of the trematode *Opistorchis felineus* (Table 1), while a single faecal sample from SL was positive for *Alaria alata* (1.5 %). Furthermore, Dicrocoelidae eggs were retrieved in two samples from AARP and were identified as belonging to *Dicrocoelium dendriticum*.

Taeniid eggs were retrieved only in eight out of 66 samples (12.1 %) and all of them were from AARP (14.5 %).

Among protozoa, the prevalence of *Sarcocystis* spp. (36.4 %) was quite high and most positive samples were from AARP, while *Cystoisospora* spp. showed a much lower frequency (6.1 %) and were found only in GL and SL sites.

The presence of *Demodex* spp. mites were observed in five samples. After morphological examination, they were identified to species level as *Demodex phylloides* in three cases, while those found in two further samples were very similar to *Demodex acutipes*.

4. Discussion

The knowledge of parasitic infections in wildlife is crucial for planning effective conservation measures, as well as to highlight transmission risk of parasites of veterinary and public health concern (Molnar et al., 2019; de Macedo et al., 2020; Stronen et al., 2021). This copro-microscopical study added new information on grey wolf endoparasite infections in the Italian Apennines, for which very few previous data were available, despite this area hosting wolf populations that have recolonized a large part of the Alps in recent decades. Our results showed that wolves living in the examined areas of central Italy are infected with various helminths and protozoa taxa, often as coinfections, some of which have zoonotic potential. However, additional parasites may have remained undetected or the prevalence of some identified parasites underestimated, especially in the case of cestodes and flukes, due to several factors, including infections caused by immature or senescent parasites (i.e. in pre- and post-patency period), uneven or irregular presence of eggs in faeces, and the small amount of faecal sample analysed (Kulmer et al., 2025; Schneider et al., 2025). In agreement with previous Italian studies (Paoletti et al., 2017; Molnar et al., 2019), a high overall prevalence of endoparasites was found (about 92 %), and the respiratory capillariid species *E. aerophilus* and *E. boehmi* were the most prevalent parasites identified in this study, with frequent coinfections (22.7 %). The wide distribution and high prevalence of these parasites in the Apennine populations we studied appear to confirm previous data from central Italy, as well as from other European areas, suggesting a high efficiency of transmission in wolves and high environmental contamination (Bindke et al., 2019; Molnar et al., 2019; Samorek-Pierog et al., 2023).

Eucoleus boehmi is a canid-specific parasite whose adults reside in the nasal turbinates and the frontal and paranasal sinuses and reported to cause rhinitis of varying severity and impaired scent in dogs (Veronesi et al., 2014b). In this study, *E. boehmi* showed the highest prevalence, especially in AARP where 76.4 % of samples were positive and whose 95 % CI of faecal prevalence differed from that of other species (Fig. 2). These results confirm recent data on grey wolves in Italy (Mariacher et al., 2015; Molnar et al., 2019) and in other European countries (Al-Sabi et al., 2018), showing a high prevalence of *E. boehmi* infections in wolves. A very high prevalence of *E. boehmi* was also reported in foxes from western Austria (Hodžić et al., 2016). In addition, *E. boehmi* was also previously reported in the red fox in Italy (Veronesi et al., 2014a; Di Cesare et al., 2015; Magi et al., 2015). Therefore, data from our work and previous reports confirm the high circulation of *E. boehmi* among wild European canids. In domestic dogs from Italy, *E. boehmi* infections have been reported as single clinical cases mainly in dogs living in central and north-western Apennine and sub-Apennine areas (Traversa et al., 2011; Di Cesare et al., 2012; Magi et al., 2012; Perrucci et al., 2014; Veronesi et al., 2014b; Cervone et al., 2017; Morelli et al., 2021), corresponding to the grey wolf expansion route in Italy (Fabbri et al., 2007). As previously suggested for red foxes (Hodžić et al., 2016), our results may indicate that the grey wolf represents an important reservoir of this respiratory nematode, and may play an important role in the transmission of the parasite between wild and domestic canids.

Differently from *E. boehmi*, *E. aerophilus* infects a range of domestic and wild animals, as well as humans (Lalosević et al., 2008; Paoletti et al., 2017). Adults are found in the epithelium of bronchioles, bronchi and trachea and may cause respiratory distress (Di Cesare et al., 2012). The red fox is considered the main reservoir of this respiratory nematode in Europe (Samorek-Pierog et al., 2023). Although frequently detected, in this study *E. aerophilus* showed an overall prevalence statistically

Table 1
Mean frequency (%) with 95 % confidence intervals of faecal prevalence (FP) of endoparasites and range of helminth eggs (EPG) and protozoan oocysts/sporocysts per gram of faeces (OPG/SPG) identified in grey wolf faecal samples, overall and in each of the four study areas from northern Tuscany, central Italy. N = total samples collected. For grey wolves from Apuan Alps Regional Park (AARP), the number of true positive samples on the total number of genotyped individuals (N = 16) and the estimated true population prevalence (TP) with 95 % confidence interval were also estimated. P-values of Fisher's Chi-square exact tests for comparison of faecal and population prevalence are included for AARP.

Endoparasites	Species	Overall (N = 66)	Apuan Alps Regional Park (AARP) (FP for N = 55; TP for N = 16)	Gherardesca Lake (GL) (N = 1)	Crespina-Lorenzana (CL) (N = 5)	Sibolla Lake (SL) (N = 5)
Nematoda	*Eucoleus boehmi*	44/66 (66.7 %, 53.9–77.8); 10-3620 EPG°	**FP**: 42/55 (76.3 %, 62.9–86.8); 10-3620 EPG° **TP**: 14/16 (87.5 %, 61.6–98.4) **P-value** = 0.4933	0/1 (0 %, 0–97.5)	2/5 (40 %, 5.3–85.3); 30-40 EPG	0/5 (0 %, 0–52.2)
	Eucoleus aerophilus	21/66 (31.8 %, 20.9–44.4); 10-3620 EPG°	**FP**: 16/55 (29.1 %, 17.6–42.9); 10-3620 EPG° **TP**: 7/16 (43.8 %, 19.7–70.1) **P-value** = 0.3636	0/1 (0 %, 0–97.5)	1/5 (20 %, 0.5–71.6); 50 EPG	4/5 (80 %, 28.4–99.5); 30-250 EPG
	Ancylostomatidae[b]	14/66, (21.2 %, 12.1–33.0); 10-510 EPG	**FP**: 7/55 (12.7 %, 5.3–24.5); 10-50 EPG **TP**: 4/16 (25 %, 7.3–52.4) **P-value** = 0.2652	0/1 (0 %, 0–97.5)	2/5 (40 %, 5.3–85.3); 90-120 EPG	5/5 (100 %, 47.8–100); 90-510 EPG
	Toxocara canis	[a]1/66 (1.5 %, 0.04–8.2)	0/55 (0 %, 0–6.5)	0/1 (0 %, 0–97.5)	0/5 (0 %, 0–52.2)	[a]1/5 (20 %, 0.5–71.6)
	Spirocerca lupi	[a]1/66 (1.5 %, 0.04–8.2)	0/55 (0 %, 0–6.5)	0/1 (0 %, 0–97.5)	[a]1/5 (20 %, 0.5–71.6)	0/5 (0 %, 0–52.2)
	Physaloptera spp.	5/66 (7.6 %, 2.5–16.8); 10–50 EPG	**FP**: 5/55 (9.1 %, 3.0–19.9); 10-50 EPG **TP**: 2/16 (12.5 %, 1.5–38.3) **P-value** = 0.6512	0/1 (0 %, 0–97.5)	0/5 (0 %, 0–52.2)	0/5 (0 %, 0–52.2)
	Angiostrongylus vasorum	[a]1/66 (1.5 %, 0.04–8.2)	0/55 (0 %, 0–6.5)	0/1 (0 %, 0–97.5)	0/5 (0 %, 0–52.2)	[a]1/5 (20 %, 0.5–71.6)
	Crenosoma vulpis	[a]1/66 (1.5 %, 0.04–8.2)	0/55 (0 %, 0–6.5)	0/1 (0 %, 0–97.5)	[a]1/5 (20 %, 0.5–71.6)	0/5 (0 %, 0–52.2)
Trematoda	*Opisthorchis felineus*	2/66 (3.0 %, 0.4–10.5); 10–50 EPG	**FP**: 2/55 (3.6 %, 0.4–12.5); 10-50 EPG **TP**: 1/16 (6.3 %, 0.2–30.2) **P-value** = 0.541	0/1 (0 %, 0–97.5)	0/5 (0 %, 0–52.2)	0/5 (0 %, 0–52.2)
	Alaria alata	[a]1/66 (1.5 %, 0.04–8.2)	0/55 (0 %, 0–6.5)	0/1 (0 %, 0–97.5)	0/5 (0 %, 0–52.2)	[a]1/5 (20 %, 0.5–71.6)
	Dicrocoelium dendriticum	2/66 (3.0 %, 0.4–10.5); 50 EPG	**FP**: 2/55 (3.6 %, 0.4–12.5); 50 EPG **TP**: 0/16 (0 %, 0–20.6) **P-value** = 1	0/1 (0 %, 0–97.5)	0/5 (0 %, 0–52.2)	0/5 (0 %, 0–52.2)
Cestoda	Taeniidae spp.	8/66 (12.1 %, 5.4–22.5); 10–1200 EPG	**FP**: 8/55 (14.5 %, 6.5–26.7); 10-1200 EPG **TP**: 3/16 (18.8 %, 4.1–45.6) **P-value** = 0.702	0/1 (0 %, 0–97.5)	0/5 (0 %, 0–52.2)	0/5 (0 %, 0–52.2)
Protozoa	*Cystoisospora* spp. (*C. canis, C. ohioensis* complex)	[a]4/66 (6.1 %, 1.7–14.8)	0/55 (0 %, 0–6.5)	*Cystoisospora canis*[a] 1/1 (100 %, 25–100)	0/5 (0 %, 0–52.2)	[a]3/5 (60 %, 14.6–94.7); *C. canis* 2/5 (40 %, 5.3–84.3); *C. ohioensis* complex 1/5 (20 %, 0.5–71.6)
	Sarcocystis spp.	24/66 (36.4 %, 24.9–49.1); 20-1330 SPG	**FP**: 23/55 (41.8 %, 28.6–55.9); 20-1330 SPG **TP**: 9/16 (56.3 %, 29.9–80.2) **P-value** = 0.7774	0/1 (0 %, 0–97.5)	[a]1/5 (20 %, 0.5–71.6)	0/5 (0 %, 0–52.2)

[a] Positive only for flotation test; °: *Eucoleus boehmi* and *E. aerophilus* eggs were counted together.
[b] Ancylostoma caninum/Uncinaria stenocephala.

Table 2
Coinfections of gastrointestinal parasites found in grey wolf faecal samples from Apuan Alps Regional Park collected between February 2020 and March 2022.

N = 55	A	B							
		N pos = 42	N pos = 16	N pos = 7	N pos = 5	N pos = 2	N pos = 2	N pos = 8	N pos = 23
		E. boehmi	*E. aerophilus*	Ancylostomidae	*Physaloptera* spp.	*O. felineus*	*D. dendriticum*	Taeniidae	*Sarcocystis* spp.
N pos = 42	***E. boehmi***	1	0.36	0.14	0.07	0.05	0.05	0.36	0.45
N pos = 16	***E. aerophilus***	0.94	1	0.25	0.12	0.06	0.06	0.19	0.56
N pos = 7	**Ancylostomidae**	0.86	0.57	1	0	0.14	0.28	0	0.71
N pos = 5	***Physaloptera* spp.**	0.8	0.4	0	1	0	0	0.2	0.4
N pos = 2	***O. felineus***	1	0.5	0.5	0	1	0	0	0.5
N pos = 2	***D. dendriticum***	1	0.5	1	0	0	1	0	0.5
N pos = 8	**Taeniidae**	0.75	0.37	0	0.12	0	0	1	0.25
N pos = 23	***Sarcocystis* spp.**	0.83	0.39	0.22	0.08	0.04	0.04	0.08	1

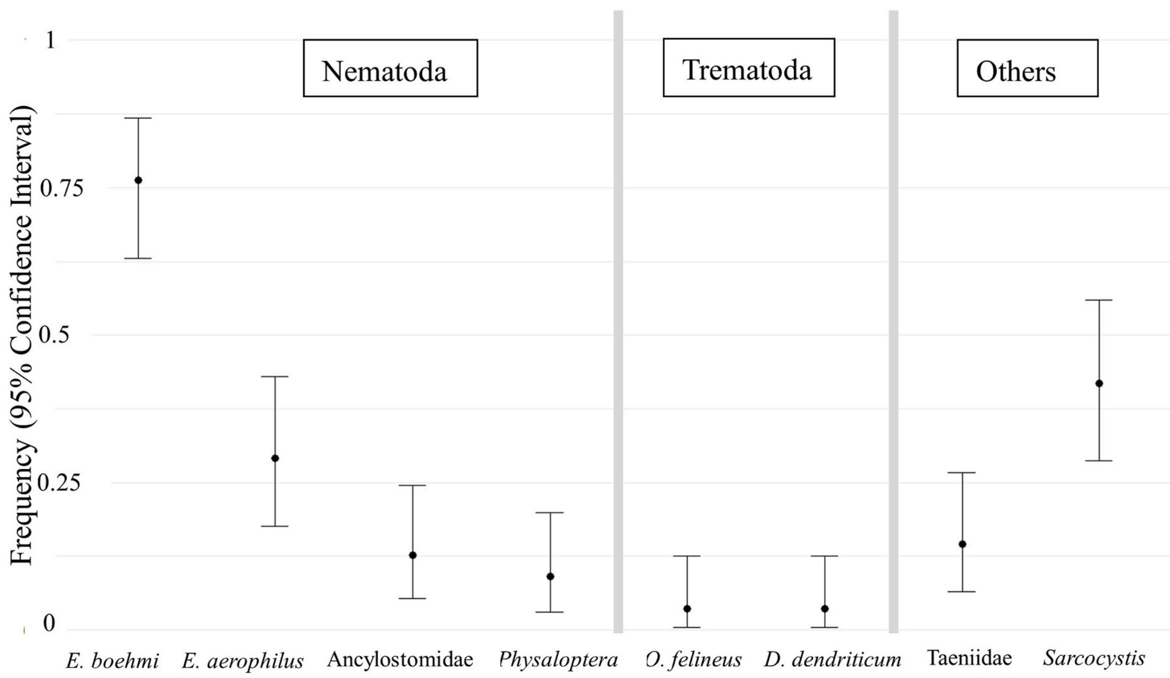

Fig. 2. Frequency and 95 % confidence interval of parasites identified in 55 grey wolf faecal samples collected in the Apuan Alps Regional Park between February 2020 and March 2022.

lower than that of *E. boehmi*. In a previous study from Italy, *E. aerophilus* was the most frequent nematode found in grey wolves (Paoletti et al., 2017). Nevertheless, the prevalence found in this latter study (21.7 %) was lower than that found in the present work (31.8 %), suggesting an important role of the wolf in the parasite transmission in the examined area.

Recent Italian studies highlighted the pathogenic potential of *A. vasorum* in grey wolves, especially juveniles (De Liberato et al., 2017; Tieri et al., 2021). *Angiostrongylus vasorum* and *C. vulpis*, a cardiorespiratory and a respiratory nematode species respectively, showed a very low prevalence in this study compared to previous reports in Italy of 66.7 % (Tieri et al., 2021) and 13.15 % (Macchioni et al., 2021), respectively. High prevalence of these two nematode species in wolves was also reported also in Portugal (Martínez-Rondán et al., 2019) and Spain (Estévez-Sánchez et al., 2022). The sample freezing before analysis, necessary to protect laboratory workers, and the diagnostic method used may have led to an underestimated prevalence of these nematodes.

Among nematodes of the digestive system, Ancylostomatids, mainly *A. caninum*, are zoonotic parasites as they may be the aetiological agent of human cutaneous *larva migrans* (Otranto et al., 2015). In humans, *A. caninum* can also cause folliculitis, myositis, and, more rarely, eosinophilic enteritis (Bowman et al., 2010). *Ancylostoma caninum* and, mainly, *U. stenocephala*, have been frequently reported in grey wolves in various Italian areas with prevalence rates ranging from about 5 % up to 50 % (Paoletti et al., 2017; Di Francesco et al., 2019; Molnar et al., 2019; de Macedo et al., 2020; Perrucci et al., 2023). This is in line with the prevalence from our research (21.2 %) and suggests that wolves may greatly contribute to the diffusion of these parasites in the examined area. Ancylostomatidae are very frequent in European grey wolves (Szafrańska et al., 2010; Al-Sabi et al., 2018; Bindke et al., 2019), likely because of the large variety of infection routes, such as ingestion of infective larvae or prey paratenic hosts, skin penetration by *A. caninum* infective larvae, and galactogenic transmission, thus increasing the opportunities for infection (Hermosilla et al., 2017; Perrucci et al., 2023).

The genus *Physaloptera* includes some gastric nematodes with an indirect life cycle with carnivores as definitive hosts and coleopterans as intermediate hosts, and have been reported as causative agents of gastritis, vomiting, and weight loss in domestic dogs but nothing is known about the pathogenic role in wolves (Ferroglio et al., 2009). These nematodes have been reported with low/medium prevalence in foxes and wolves in the north-western regions of Italy (Ferroglio et al., 2009; Magi et al., 2015; de Macedo et al., 2020; Perrucci et al., 2023) and in foxes in other European countries (Segovia et al., 2004; Vergles Rataj et al., 2013). Prevalence of *Physaloptera sibirica* (9.5 %) previously

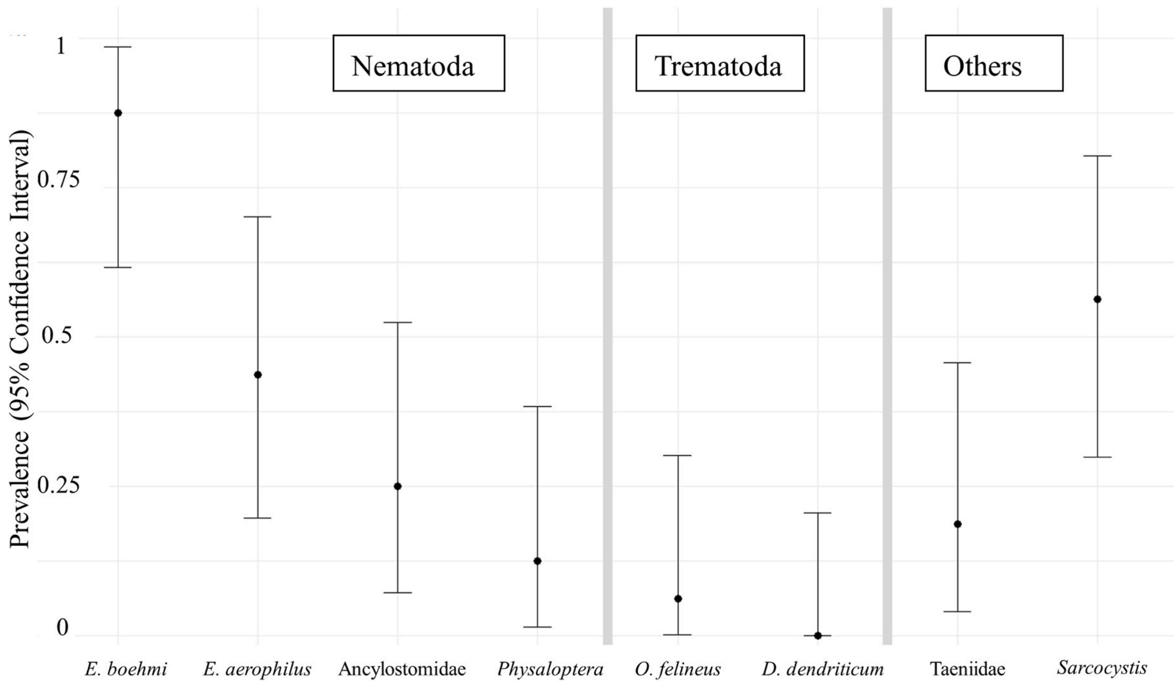

Fig. 3. True prevalence and 95 % confidence interval of parasites identified in 16 individually genotyped grey wolves from Apuan Alps Regional Park.

reported in wolves in northern Italy (de Macedo et al., 2020) is similar to that of *Physaloptera* spp. in this study (7.6 %).

Differently from gastrointestinal nematodes reported above, *T. canis* and *S. lupi* showed a very low prevalence (1.5 %), only found in a single sample from SL and CL, respectively. *T. canis* is one of the main aetiological agents of human toxocariasis worldwide, causing ocular and visceral larva *migrans* in humans (Smith et al., 2009; Chen et al., 2018) and has been previously reported in grey wolves with a prevalence ranging from 4.3 % to 10.25 % in Italy (Paoletti et al., 2017; de Macedo et al., 2020; Macchioni et al., 2021; Perrucci et al., 2023), and from 2.8 % to 13.0 % in other European countries (Čabanová et al., 2017; Hermosilla et al., 2017; Bindke et al., 2019). Since this ascarid species is generally more prevalent in younger animals (less than 6 months of age), this could indicate that the faecal samples analysed in this study belonged mainly to adult individuals, leading to an underestimate of *T. canis* true prevalence. *Spirocerca lupi* is a spirurid nematode whose adults localize within oesophageal nodules and females shed eggs that can be found in the faeces of infected canids (Rojas et al., 2017). *Spirocerca lupi* infection is infrequent in free-roaming wolf packs and is generally reported with low prevalence, as emerged in our study. Indeed, in previous European studies this nematode was found in 11.5 %, 7.7 % and 0.8 % of examined faeces from Poland (Szafrańska et al., 2010), Sweden (Al-Sabi et al., 2018) and Slovakia (Čabanová et al., 2017), respectively.

Cestodes of the Taeniidae family have an indirect life cycle with definitive and intermediate hosts and include several species of zoonotic potential (Jones and Pybus, 2008). In Italy, the grey wolf has been reported as definitive host of different taeniid species (Poglayen et al., 2017; de Macedo et al., 2020; Macchioni et al., 2021; Bariselli et al., 2023; Cafiero et al., 2025), of which *Taenia serialis*, *Taenia multiceps*, *Echinococcus granulosus sensu lato* and *Echinococcus multilocularis* are zoonotic. Molecular tools are needed for genus and species differentiation since egg morphology is not a distinctive feature for species identification (Thompson, 2017). Taeniid eggs found in 14.5 % of samples from AARP in this study, were identified as belonging to three taeniid species, namely *E. multilocularis*, *Taenia hydatigena* and *Taenia krabbei* in a further study by us (Cafiero et al., 2025).

In the present study, trematode eggs morphometrically referrable to *O. felineus* were identified for the first time in Italian wolves, (AARP, 3.6 %). To the best of our knowledge, *O. felineus* has been reported previously in European grey wolves only in Croatia (Hermosilla et al., 2017). *O. felineus* is a liver fluke distributed in Europe and Asia, and it was described for the first time in the nineteenth century in domestic cats and dogs from Pisa, Italy (rev. in Pozio et al., 2013). *Opistorchis felineus* was occasionally reported in red foxes in many European countries (Schuster et al., 2003; Segovia et al., 2004; Miterpáková et al., 2009), including Italy (Macchioni et al., 2012; Pozio et al., 2013), and in cats of central Italy with an overall prevalence of 46.4 % (De Liberato et al., 2011). *Opisthorchis felineus* infection of canid, felid and human definitive hosts occur after ingestion of raw freshwater fish from rivers and lake basins or deriving from anthropogenic waste (Pozio et al., 2013). Therefore, data from this study reinforce previous observations on the alimentary flexibility of European grey wolves (Hermosilla et al., 2017).

Although *A. alata* was a frequent grey wolf parasite found in Poland (Szafrańska et al., 2010; Karamon et al., 2024) and in Germany (Bindke et al., 2019), this trematode species showed a very low prevalence (0.3 %) in Croatian free-living wild wolves (Hermosilla et al., 2017). In Italy, *A. alata* has been reported with low prevalence in wild boars and red foxes in northern Italy (Fiocchi et al., 2016; Gazzonis et al., 2018), and in few wolves from Abruzzo, Lazio and Molise National Park, central Italy (Molnar et al., 2019). Therefore, the detection of *A. alata* in a faecal sample collected in SL in this study confirmed the presence of this trematode species in the grey wolf and wildlife in Italy. In the life cycle of *A. alata,* freshwater snails and amphibians act as the first and second intermediate hosts, respectively, whereas reptiles, rodents, wild boars, and other vertebrates, including humans, can act as paratenic hosts after feeding on second intermediate hosts or paratenic hosts (Moehl et al., 2009). Therefore, as in the case of *O. felineus*, the presence of freshwater riverine habitats occurring in our study areas is also crucial to the life cycle of this parasite.

Both *O. felineus* and *A. alata* are potential zoonotic agents causing opisthorchiasis and alariosis, respectively (Moehl et al., 2009; Mordvinov and Furman, 2010). Despite most cases of humans opisthorchiasis are asymptomatic or disappear in a few days, severe and chronic infections may affect the pancreas and hepatobiliary system (Mordvinov and Furman, 2010). Clinical signs of human alariosis range from low-grade respiratory and cutaneous symptoms to neuroretinitis and anaphylactic shock (Moehl et al., 2009). Nonetheless, few data are available about the role of wild canids in spreading these parasitic zoonoses (Otranto et al., 2015).

Among flukes, Dicrocoeliidae eggs were detected in two samples, and they were identified as the lancet liver fluke *D. dendriticum*. This finding confirms previous observations in grey wolf populations in another natural area of Italy, namely the Abruzzo, Lazio and Molise National Park (Molnar et al., 2019). Here, *D. dendriticum* was regarded as a pseudoparasite and its presence in wolf faeces was considered a result of predation on infected prey. Indeed, *D. dendriticum* is prevalent in domestic and wild ruminants in the area examined in this study (Secchioni et al., 2016; Scarcelli et al., 2024). However, dogs and cats can be also infected by this liver fluke species, as well as wild brown bears (Reljić et al., 2017; Tuska-Szalay et al., 2024). Therefore, the possibility of *D. dendriticum* infections in wolves cannot be completely excluded.

Among protozoa, *Cystoisospora* spp. (*C. canis* and *C. ohioensis*-like oocysts) were identified only in GL and SL with a low prevalence (6.1 %). In wolves, coccidian infections (*Cystoisospora* spp.) are prevalent especially among pups, in which they can cause haemorrhagic enteritis, diarrhoea, growth reduction and death (Mech and Kurtz, 1999; Molnar et al., 2019).

The relatively high prevalence of *Sarcocystis* spp. found in this study is not surprising, since these protozoa have a two-host life cycle with mainly carnivores and humans as definitive hosts and herbivores and omnivores as intermediate hosts. Roe deer, red deer and wild boars sharing with wolves the habitat in the study areas may act as intermediate hosts of several species infecting European grey wolves (Guardone et al., 2022), namely *Sarcocystis capreolicanis*, *Sarcocystis linearis* and *Sarcocystis miescherania*, respectively. However, *Sarcocystis* species cannot be distinguished based on morphology of oocysts/sporocysts found in wolf faeces, and molecular diagnostic methods are needed (Lesniak et al., 2017).

Adult stages of *Demodex* spp. were detected in five samples from AARP. Based on morphological and metrical features, these mites were identified as *Demodex phylloides* in three samples. *Demodex phylloides* is a parasite specific of domestic pigs and wild boars (Fryderyk and Izdebska, 2001; Schwarz et al., 2024), that could have been ingested after predation, since wild boar is one of main prey in grey wolf diet across central Italy (Stählberg et al., 2017; Bassi et al., 2020). Based on morphometrical features, it was assumed that mites found in the remaining two samples could be *Demodex acutipes,* a rare parasite of European red deer (*Cervus elaphus*) (Izdebska and Fryderyk, 2012). Nonetheless, this species was never reported in Italy before.

5. Conclusions

The data obtained in this study contribute to the knowledge on wolf parasites in the studied area, as well as on the wolf role in the transmission of some parasitic species we detected. Specifically, the results showed a high faecal and true prevalence of endoparasites, especially respiratory capillariids, *Sarcocystis* spp. and hookworms. Potentially zoonotic species were also detected, such as *E. aerophilus*, *O. felineus*, *A. alata*, *T. canis*, *A. caninum* and taeniids, whereas other identified endoparasites may negatively affect the health of infected wolves, namely *A. vasorum* and *Cystoisospora* spp. The potential role of grey wolves in the circulation and transmission to wild and domestic canids of *E. boehmi* across the examined area was particularly highlighted. Therefore, data from this study may represent a baseline for future studies on wolf parasites.

CRediT authorship contribution statement

Salvatore Andrea Cafiero: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Luca Petroni:** Writing – review & editing, Investigation. **Luca Natucci:** Investigation. **Luigi Casale:** Writing – review & editing, Investigation. **Marta Raffaelli:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Debora Baldacci:** Investigation. **Alessia Di Rosso:** Writing – review & editing, Investigation. **Chiara Rossi:** Methodology, Investigation, Formal analysis. **Adriano Casulli:** Resources, Funding acquisition. **Alessandro Massolo:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Heidi Christine Hauffe:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Stefania Perrucci:** Writing – review & editing, Writing – original draft, Supervision, Resources, Data curation, Conceptualization.

Data availability statement

The data presented in this study are available on request from the corresponding author.

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Declaration of competing interest

All authors declare that there are no competing interests.

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