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Giardia, Cryptosporidium and *Eimeria* infections in alpine wild ungulates: epidemiological investigation and management implications

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Abstract

Monitoring and management of wildlife health are of increasing importance in the last decades, first of all considering that more of 70% of emergent zoonoses are host in wild animals. Moreover the public health issue must be seen also in accordance with the European food hygiene regulations (Reg. EC 852, 853, 854/2004), that make hunters primary producers intended to auto-consumption and/or commercialization. We have to consider wildlife health value also in relation to possible interactions with livestock and to population dynamics and biodiversity preservation. We focused our attention on protozoa, parasites with the highest success spreading, in particular on *Giardia*, *Cryptosporidium* and *Eimeria* in chamois (*Rupicapra rupicapra rupicapra* and *Rupicapra pyrenaica ornata*), red deer (*Cervus elaphus*) and roe deer (*Capreolus capreolus*). Faecal samples were collected, in the biennium 2013-2014, from Lepontine Alps and from central Apennine. Through immunofluorescence emerged *Giardia* prevalence of 5,82% in *R. r. rupicapra*, and of 1,85% in *R. p. ornata.* Positive samples were confirmed by *q* PCR and end-point PCR, with identification of zoonotic assemblage A and livestock assemblage E. A parallel survey was conducted through immunoenzimatic methodology. *Giardia* showed prevalences of 4,5%, 2,5% and 8,4% while *Cryptosporidium* of 1,2%, 0,5% and 3,4% respectively in chamois, red deer and roe deer. Positive samples were subjected to molecular investigations. Assemblage A of *Giardia* was detected in chamois and red deer, suggesting a potential zoonotic risk, although rather low. Assemblage E was detected in chamois, highlighting a trasmission of this parasite between livestock and wildlife. Concerning *Cryptosporidium*, immunoenzimatic positivities haven't been confirmed by PCR. Roe deer shows higher prevalences for both protozoa, statistical analyses about *Giardia* infection highlight a higher probability of calves being infected than adult, and higher significant probability to contract the infection in animals culled at lower altitudes, probably due to impact originated by human activities. Finally faeces were analysed by FLOTAC methodology in order to quantify emissions of coccidian oocysts and nematode eggs. *Eimeria* shows prevalence of 81,1% in chamois, 46,1% in red deer and of 43,4% in roe deer. Overall prevalences for gastro-intestinal nematodes are 76,4% in chamois, 57,4% in red deer and 67,1% in roe deer. Statistical analyses were carried out to define the epidemiological role of coccidian infections in wild ungulates in alpine context. Data collected about coccidia and gastro-intestinal nematodes don't allow a correct definition of simultaneous parasitosis, considering nematodes possibility to infect several hosts. About that *Eimeria* represents a useful model to better understand interaction among pathogen, host and environment at intraspecific level,

because of its strict host-specifity At the moment comparison between data about all protozoa investigated and alpine data census don’t suggest any impact on population dynamics. However it can not be completely excluded because of limits related to period of sampling that not always correspond to critical period for these infections in wild ungulates. We need to increase sampling season, compatibly with weather conditions, and to produce a data series about emissions of oocysts to better understand the eco-epidemiological role of protozoa in wildlife.

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Chapter 1

Introduction

1.1 One World, One Health: why monitoring wildlife

Man, animal populations and ecosystem are more and more closely related as a result of a relevant increase of human activities because of modernization and industrialization; increasing human encroachment into wildlife territory, at different levels, cause significant wildlife habitat fragmentation and loss of biodiversity (Daszak et al. 2001, Waltner-Toews 2009, Dhama et al. 2013). Increase in international trade and travel, global climate change, habitat destruction, ecotourism, changes in ecosystem and biodiversity have resulted in exposure of humans, as well as animals, to new pathogens to which they had never been exposed before, requiring proper veterinary attention (Dhama et al. 2013). All these changes constitute relevant risk factors for diseases in such populations resulting in the emergence and re-emergence of diseases, including zoonoses (Taylor et al. 2001, Myers and Patz 2009). Therefore an interdisciplinary approach to prevention, surveillance and disease control is desirable in the perspective of ecosystem health (Nielsen 2005), as assessed by recent guidelines of "*The Manhattan Principles on One World, One Health"* (FAO-OIE-WHO, 2010). Wildlife health become an essential component of global disease monitoring and mitigation, related also to the increase of international wildlife and bushmeat trade, in order to integrate biodiversity conservation perspectives and human needs and to develop solutions to infectious disease threats. One Health requires multidisciplinary efforts at global, national and local levels, and engages veterinarians, medical doctors, biologists, technicians and professionals in different ecological contexts to address the world most challenging health concerns (Gibbs and Anderson 2009).

In this context we have to give more attention to wildlife, taking also into account that more than 70% of emerging zoonoses are hosted in wild animals (Jones et al. 2008). A recent example is represented by the severe epidemic of Ebola virus disease occurring in West Africa where fruit and insectivorous bats have been recorded as potential sources for Ebola virus outbreaks (Groseth et al. 2007, Mari Saez et al. 2015).

Leading causes for the occurrence of zoonoses from wildlife could be represented by growth of human population with consequent invasion, fragmentation and loss of wildlife habitats (Wilcove et al. 1986, Daszak et al. 2001) and environmental changes that may affect the dynamics of disease. A significant example is represented by changes occurred in Amazon rainforest, where deforestation altered the cycles of parasitic diseases (e.g. filariosis, leishmaniasis), because of a change in several ecological niches and consequent unbalanced populations of wildlife reservoirs (Confalonieri et al. 2014).

Increase and changes in agricultural practices, like domestication of wild animals, leading to reemerging zoonoses such as bovine tuberculosis in deer populations kept in captivity (Wilson 2002) and brucellosis in wild boar (Godfroid et al. 2005). Furthermore any movement of wild animals, intended to hunting repopulation or reintroduction, could introduce

new pathogens (Chomel et al. 2007, Dhama et al. 2013). For example in 1970's in the mid-Atlantic states rabies was introduced during a relocation of hunting pens with raccoons trapped in rabies endemic zone of the southern United States (Woodford and Rossiter 1993). Animals trade, in particular illegal trade not exposed to sanitary controls, can also be a possible source of zoonoses. In March 1994 psittacosis developed in several customs officers in Belgium (De Schrijver 1998). Ecotourism increases the risk that people participating in activities such as safaris, adventure sports and extreme travels will contact pathogens uncommon in industrialized countries (Chomel et al. 2007, Dhama et al. 2013). Just think to the most commonly encountered rickettsial infection in travel medicine: African tick bite fever caused by *Rickettsia africae* transmitted by ungulate ticks of the *Amblyomma* genus (Ericsson et al. 2004).

The opportunities for contact between humans and wild animals are numerous, corresponding to different pathogens transmission mode: by aerosol as bacteria of the genus *Brucella* (Godfroid 2002) and the agent of Q fever, *Coxiella burnetii* (Castillo et al. 2010). Other pathogens can be transmitted by means of direct contact or in consequence of little cutaneous injury as *Francisella tularensis*, the causative agent of tularemia, through skin contact with an infested, diseased, or dead hare or rodent (Kruse et al. 2004). Environmental pollution can also be a source of human infection, just think to protozoan dissemination as *Cryptosporidium* or *Giardia* (Smith et al. 2007) or bacteria as *Listeria monocytogenes* (Oevermann et al. 2010). Moreover the public health issue must be seen also considering the importance of foodborne zoonoses, overall in accordance with the European food hygiene regulations (Reg. EC 852, 853, 854/2004) that make hunters primary producers of game intended to auto-consumption or commercialization. For example human trichinosis occurred in association to consumption of wild boar (*Sus scrofa*) (García et al. 2005, Pozio 2007), or acquired ocular toxoplasmosis in deer hunters (Ross et al. 2001); actually DNA presence of *T. gondii* in a chamois carcass, highlights importance of monitoring and preventing different pathogens related to wildlife meat consumption or manipulation (Formenti et al. 2015a).

We have to consider wildlife health value also in relation to possible interactions with livestock, in fact it became increasingly apparent that wild species can act as a reservoir, vector or as casualties of diseases transmissible to domestic animals (Artois et al. 2001). Disease is strongly associated with environmental factors; for a well defined target population, the study of a disease should be related to the study of interactions between environment, pathogens and human activities, so we need to define the epidemiological reservoir, that change according to different parameters, actually for several pathogens we have yet to discover the real reservoir (Martin et al. 2011). We could assume that all susceptible host populations epidemiologically connected, directly or indirectly, to the target population for a given pathogen, could potentially constitute all or part of the reservoir (Haydon et al. 2002).

Categorisation of host species isn't definite: spillover hosts have dynamic role and they may become a reservoir and consequently a spillback to domestic animals, highlighting a disease flow between wildlife and either domestic animals

or humans in both directions (Haydon et al. 2002, Morgan et al. 2004, Thompson et al. 2009, Martin et al. 2011, Thompson 2013). Several significant examples of these complex interactions are represented by maintenance of *Mycobacterium bovis* in wild populations and consequently in livestock: this is the case of red deer in France and New Zealand (Zanella et al. 2008, Nugent 2011, Barron et al. 2013) and of the badgers in England and Ireland (Corner 2006, Delahay et al. 2007).

Different factors can explain the constantly increasing interactions between wild and domestic animals. As well as for zoonoses issues, a major parameter is the growing human population. In most European countries, reduction and fragmentation of natural spaces are the main anthropogenic factors associated with the emergence of diseases in wildlife, large populations of wild ungulates are in fact concentrated in small delimited areas because of high human distribution (Acevedo-Whitehouse and Duffus 2009, Martin et al. 2011). In this context pastures can become overlapping areas where the transmission rate of infectious diseases is the highest (Van Campen and Rhyan 2010), often implicating severe economic loss due to drug treatments or even cases of mortality among more susceptible subjects.

Finally wildlife health issues should be considered in relation to population dynamics and biodiversity preservation (Daszak et al. 2001, Deem et al. 2001, Pedersen and Fenton 2007). There are newly discovered pathogens that have resulted in population decline or global extinction of several species of high ecological value (Sleeman 2013): *Batrachochytrium dendrobatidis* causes a cutaneous fungal infection of amphibians and is linked to decline of them globally (Kriger and Hero 2009) and the white nose syndrome (*Pseudogymnoascus (Geomyces) destructans*) has caused dangerous decline of bat species in North America and India (Thogmartin et al. 2012, Reeder and Moore 2013).

However, considering all factors about a wide concept of health mentioned before, a change in "disease ecology" emerge: we can lose endemic stability and the balance among pathogen, host and environment can fail (Deem et al. 2001). It is not possible to draw a clear and unequivocal distinction between pathogens and non-pathogens, pathogenicity can be due to the immune response to the pathogen rather than to the pathogen itself (Ryser-Degiorgis 2013). Moreover not always we should tackle any microrganisms, indeed the extent of a pathogen influences community structure and biodiversity via host population regulation. Loss of biodiversity occurs when disease is introduced into naive population (Daszak et al. 2000).

In this context monitoring free ranging wild animals health is even more difficult for several reasons. Indeed signs of infection can be difficult to assess because they are often not visible, population size cannot always be estimated with confidence and also rates of morbidity and mortality may remain unclear (Artois et al. 2001). In particular sub-clinical diseases can have an impact on animal well-being, affecting individual hosts by reducing growth rates or fecundity, increasing metabolic requirements, or changing patterns of behavior (Ryser-Degiorgis 2013). Therefore wildlife diseases are observed through passive surveillance in sick or dead animals accidentally found and active surveillance

should support these investigations (Guberti et al. 2014). A monitoring plane is necessary to survey wild animal health but it must be conceived in order to develope not-invasive methods and minimize human impact on free-ranging populations. This is even more important considering that several species are difficult to be contacted because of either their ethological behavior and the impossibility to reach them. Anyway we can monitor these populations by direct or indirect (e.g. by means of photo traps) observation, in order to detect any signs of disease, and then we can collect different biological matrices to carry out several type of investigations, e.g. hair or pens for molecular analyses, bird pellets to study alimentation or faeces for parasitosis monitoring or molecular investigation. Obviously these methods cannot be applicable to every situation, but they assume an essential role in contexts where hunting isn't allowed or in monitoring of protected species.

1.2 Parasite in wildlife: the success of protozoa

Parasites are organisms that naturally developed different strategies of survival and spread in the environment. In particular some pathogens are frequently specialised, infecting only one or a few related species, usually with limited effects on these hosts because of their good health conditions (Malpica et al. 2006, Gortázar et al. 2007). Many parasites are defined as multi-host pathogens infecting a wide range of subjects belonging to different taxonomic groups (Malpica et al. 2006) performing direct or indirect lifecycle with intermediate hosts. In true multihost situations, pathogen can persist in either host population independently of the absence of the other (Gortázar et al. 2007). As well as parasites can affect more hosts, most host animal populations encounter a large number of different pathogen species. Multiple infections may have important consequences for host-pathogen coevolution (Woolhouse et al. 2002), in particular dynamics of microparasites in multiple infected hosts may have relevant consequences in the evolution of their virulence (May and Nowak 1995).

The multi-host pathogens are characterized by the highest spread capacity, and they could be responsible for emerging infectious disease outbreaks in humans, livestock and wildlife (Woolhouse 2002) where they spatially overlap. Parasites shared among different host species assume not so much importance taken individually, but when they act together, causing economic losses in regard to livestock, and influencing population dynamics in wildlife (Gortázar et al. 2007).

Several classes of protozoa are defined multi-host pathogens, affecting humans and both livestock and wildlife. For example *Sarcocystis* is a zoonotic apicomplexan protozoa that needs an intermediate host, usually herbivores or omnivore, while definitive host is usually a carnivore. Humans are definitive host, just in sporadic cases aberrant intermediate host (Lau et al. 2014, Fayer et al. 2015), and become infected through ingestion of raw or undercooked meat (*S. hominis* e *S. suihominis*). Wild ungulates, as well as domestic ruminants, can be intermediate host and in this phase of lifecycle infections are usually asymptomatic, but if large numbers of sporocysts are ingested by pregnant or young animals, clinical disease and death can occur related to abortion, and hemorrhaging of mucosal surfaces (Duncan et al. 2000, Khan and Evans 2006, Bregoli et al. 2014). *Balantidium coli* is another zoonotic protozoa; infection come from ingestion of cysts in polluted water or food. Pigs are natural reservoir of this parasite, but a recent study demonstrated a potential role of wild boar in the epidemiology of human balantidiasis; in wild boars, as well as in pigs, latent, asymptomatic or invasive, leading to diarrhoea infection may occur (Solaymani-Mohammadi et al. 2004). Toxoplasmosis also represents a considerable issue for public health, in particular regard to pregnant women who become infected through consumption of undercooked meat or ingestion of food or water contaminated with sporulated *T. gondii* oocysts excreting in faeces by the definitive host, wild and domestic felids (Gauss et al. 2006). Humans and a

wide range of animals represent intermediate hosts. In livestock *T. gondii* can affect reproductive performance, with obvious economic consequences. Recent studies about red deer highlight negative effects on foetal development, demonstrate a potential indirect impact of the protozoa on red deer reproduction and consequently on population dynamics (Formenti et al. 2015a).

Concerning wildlife health and protozoa we have also to consider *Eimeria*, *Giardia* and *Cryptosporidium*, most common in domestic animals (Olson et al. 2004, Thompson et al. 2008). These two last protozoa in particular can be zoonotic, and not needing an intermediate host in lifecycle, they have a spreading success significantly higher than other parasites, also considering their small size, a faecal-oro cycle and survival for weeks or even months in the environment (Betancourt and Rose 2004). Furthermore, the infective dose is very low since a very small number of cysts and oocysts can cause infection (Skotarczak 2010).

In particular *Giardia* and *Cryptosporidium* can seriously affect young children, pregnant women and persons with a severely weakened immune system, causing even mortality cases (Mor et al. 2010, Lendner et al. 2011, Nissapatorn and Sawangjaroen 2011, Tali et al. 2011). *C.parvum* , *C. hominis* and *G. duodenalis* are mainly involved in human enteric infections (Hunter and Thompson 2005, Benamrouz et al. 2012, Certad et al. 2012).

Epidemiological context is very complex: numerous host species, either wild and domestic, are susceptible to these infections (Feng 2010) and could have a role in maintenance and spread of these protozoa in the environment, above all in regard to ungulates, with pollution of streams, lakes and even urban water supply (ten Veldhuis et al. 2010). Presence of *Cryptosporidium* and *Giardia* is well documented in livestock (O'Handley and Olson 2006, Geurden et al. 2008, Santín and Fayer 2009) with severe consequences on juvenile classes (Díaz et al. 2015); instead little is known about the presence of these protozoa in wildlife, above all from a molecular perspective, necessary to understand their epidemiological role.

Cryptosporidium prevalence in wild ungulates in Norway and Spain has been stated up to 43% in fallow deer (*Dama dama*), and ranging from 4% to 6% in other cervids as reindeer (*Rangifer tarandus*), red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*) and moose (*Alces alces*); meanwhile *Giardia* prevalence has been stated up to 13% in moose, 2% in red deer, 16% in roe deer, 7% in reindeer and 1,5% in wild boar (*Sus scrofa*) (Hamnes et al. 2006, Castro-Hermida et al. 2011). Actually few studies have characterized *Cryptosporidium* at molecular level in wild ungulates. White-tailed deer is the only free-ranging species in which *Cryptosporidium* have been genotyped, and the cervine genotype and *C. parvum* were the major species/genotypes reported (Perz and Le Blancq 2001, Feng 2010). Other genotyping studies have been performed mostly with captive cervids, and *C. parvum* has been found mostly in these animals, including red deer and fallow deer (Alves et al. 2003). Few studies had also addressed *Giardia* characterization issue in wild ungulates, zoonotic and livestock assemblage have been reported in cervids (fallow deer, roe deer and red

deer) (Lalle et al. 2007, Solarczyk et al. 2012, García-Presedo et al. 2013) and recently in alpine chamois (*R. r. rupicara*) for the first time (De Liberato et al. 2015).

About *Eimeria*, several species of this parasite are involved in different ruminant hosts, but there is no cross infection due to the strict host specificity. In livestock coccidiosis is of great economic importance because of phenomena of clinical deseases (diarrhoea and possible mortality) and subclinical infections (severe weight loss and deterioration of general body condition) (Wang et al. 2010, Andrews 2013). Clinical coccidiosis usually occur during massive ingestion of sporulated oocysts in infected environment - sporulated oocysts show a great resistance surviving several months or even more than a year - or in relation to a lowered resistance of the host (Chartier and Paraud 2012). Prevalence and intensity of *Eimeria* shedding are highest in kids, lambs and calves younger than 4-6 months (Mundt et al. 2005, Tafti and Mansourian 2008).

Previous surveys have been carried out about eimeriosis of wild ungulates in Italy and other european countries, mostly descriptive investigation that underline prevalence data and morfological identification (Pilarczyk et al. 2005, Kowal et al. 2015).

On the whole concerning protozoan infections in free ranging wild ruminants the need arises to deepen their epidemiological role also through molecular characterisation and analyses of intrinsic risk factors that may favour the infection.

1.3 Aims of the study

The activity was aimed to describe wild ungulates epidemiological role in the spread and maintenance of *Giardia*, *Cryptosporidium* and *Eimeria* in the alpine context, related to any zoonotic, zoo-economic and conservationistic issue.

Considering Italian context, in the latest decades wild ungulates population growing has been surveyed (Carnevali et al. 2009). At the same time a marked decrease of agricultural activities has caused an increase of forest range, resulting in a pastures reduction either in terms of areas and in terms of quality, inducing wild and domestic ungulates in search of better grazing lands, then facilitating their interaction.

In alpine territory in particular, the situation is more complex due to the dramatic anthropization caused by rising urbanization, facilities building (e.g. power lines, ski-lifts, cableway) and more and more frequent presence of tourists and sportsmen. All these factors can induce a state of stress, allowing possible disease development. Although probably in wild animals great parasitic charges don't occur (Stancampiano et al. 2003) and despite the host-specificity of *Eimeria*, possibility of coinfection with other macro or microparasite, due to interaction with livestock, must be taken into account with respect to worsening of the pathogenic effect of protozoan infection (Taylor 2009). The importance of studying the trend of these parasitosis lies also in lack of clinical symptoms in wildlife (Stancampiano et al. 2003). In certain conditions, at least in domestic ruminants, coccidiosis can be characterised by sudden mortality in particular amongst young animals from 2 to 4 months old, usually they occur subclinically with weight loss, dehydration and worsening of animal general condition (Chartier and Paraud 2012).

In the Alps specific surveys have never been performed about *Cryptosporidium* and *Giardia* presence and molecular characterization. They have been spotted mainly during generic coprological surveys, e.g. Alpine ibex (*Capra ibex*) in Switzerland (Marreros et al. 2012). At the same time on italian side *Cryptosporidium* has been detected in two chamois during a summer survey for a thesis in Lepontine Alps, suggesting moreover a role in decrease of chamois yearling population within the Natural Park of Alpe Veglia – Alpe Devero (Trogu 2012).

Increase of wild ungulates populations has unquestionable naturalistic and socio-economic value, in terms of cultural enrichment, ecotourism increase, hunting activity and consequent any production and promotion of typical products from Alps and Apennines. This increasing attention to wildlife opens new epidemiological scenery and interests public opinion in order to defend and guarantee animal welfare and human health.

Starting from earlier acquired knowledge (Trogu 2012), a the study was carried out in two contiguous areas with different population management: a hunting district (VCO2-Ossola Nord) and a protected area (Alpe Veglia - Alpe Devero Natural Park) where hunting is prohibited, and an additional area in Abruzzo, Lazio and Molise Naturl Park.

Alpine wild ruminants are target species of the investigation: chamois (*R. r.rupicapra* and *R. p. ornata*), red deer (*Cervus elaphus*) and roe deer (*Capreolus capreolus*). Considering the hunting ban, within protected area it arises the need to employ not-invasive methods, therefore faeces are an useful matrix to perform parasitological investigations.

In particular purposes of the study are:

1. To define *Giardia, Cryptosporidium* and *Eimeria* prevalences in host species within the study areas;
2. To approach further molecular analyses of *Cryptosporidium* and *Giardia* positive samples to genotyping protozoa and then to define any zoonotic risk and epidemiological context in regard to interaction with livestock;
3. To carry out copromicroscopic surveys to determine *Eimeria sp.* (OPG) in the same wild ungulates, in different classes (host-species, age and gender), in order to define any possible factor, related either to animals or to environment, influencing coccidian infections.

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Chapter 2

Giardia duodenalis in Alpine (*Rupicapra rupicapra rupicapra*) and Apennine (*Rupicapra pyrenaica ornata*) chamois

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Giardia duodenalis in Alpine (*Rupicapra rupicapra rupicapra*) and Apennine (*Rupicapra pyrenaica ornata*) chamois

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Abstract

Background: Although chamois *Rupicapra* spp. are the most abundant mountain ungulates in Europe, no data are available on the presence of *Giardia duodenalis* infecting these species.

Methods: A total of 157 fecal samples from Alpine *Rupicapra rupicapra rupicapra* and Apennine *Rupicapra pyrenaica ornata* chamois were tested for the presence of *G. duodenalis* by immunofluorescence test, quantitative Real Time PCR and end-point PCR for genotype characterization.

Results: *G. duodenalis* was detected in *R. r. rupicapra* and *R. p. ornata*, with a percentage value of 4.45 (5.82 and 1.85 %, respectively), and a cyst burden of up to 31,800 cysts/g of feces. Assemblages A/AI and E were identified in *R. r. rupicapra* and assemblage A/AIII in *R. p. ornata*.

Conclusions: The present study represents the first record of *Giardia duodenalis* in *Rupicapra* spp., suggesting that these wild bovids can play an epidemiological role in environmental contamination and transmission of both zoonotic and non-zoonotic genotypes.

Keywords: *Giardia duodenalis*, *Rupicapra rupicapra rupicapra*, *Rupicapra pyrenaica ornata*, IF, *q*PCR, *end-point*-PCR

Background

The flagellate *Giardia duodenalis* is one of the most common intestinal parasites in humans and several animal species worldwide [1, 2]. At present, eight assemblages have been genetically recognized (A-H), which differ in host specificity: zoonotic assemblages A and B infect humans and a wide variety of domestic and wild mammals; assemblages C and D are typically isolated from dogs; assemblage E is associated with hoofed livestock; assemblage F infects cats; assemblage G infects rats [3], and assemblage H infects marine mammals (pinnipeds) [4]. It is now believed that at least some of these assemblages should be considered "true species" [5, 6].

Given this great genetic heterogeneity, it is hard to determine the role of animals as a source for human infection, and *viceversa* [7]; this is possible only by performing detailed genetic analysis [8, 9], even as far as the sub-assemblage level. While the role of domestic animals (pets and livestock) in *G. duodenalis* epidemiology has been thoroughly studied, wild animals have only recently been considered as having a potential role. In addition to being a possible source of infection for humans, wild animals can be endangered by the spill-over of parasites from domestic animals and even people [10], especially in the case of small populations which are important for wildlife conservation [11].

G. duodenalis has been recorded in wild ungulates wordwide [8, 9, 12–15]. Most of these records refer to cervids, in which the presence of zoonotic and non-zoonotic genotypes have been documented (reviewed by [6]).

The chamois (Artiodactyla: Bovidae) is the most abundant mountain ungulate in Europe and the Near East. Two species are recognised in the genus *Rupicapra*: the Northern chamois, *Rupicapra rupicapra*, with seven subspecies, including the Alpine chamois *R. r. rupicapra*, and the Southern chamois, *Rupicapra pyrenaica*, with

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three subspecies, including the Apennine chamois *R. p. ornata* [16, 17].

Despite the geographical abundance of *Rupicapra* species in Europe, no data are available on the presence of *G. duodenalis* infecting chamois. This study aimed to determine the presence of *G. duodenalis*, and to quantify and characterize isolates from two subspecies of chamois: *R. r. rupicapra* living in northern Italy (Alps) and *R. p. ornata*, living in central Italy (Apennines).

Methods

Study areas, animals and collection of fecal samples

The study took place in three areas of Italy. The first is in the Lecchesi Alps and Pre-Alps, a hunting territory in Lombardy region, with an area of 253 km^2 (45°59'N, 9° 32'E), ranging from 300 to > 2000 m a.s.l. Here the *R. r. rupicapra* population in 2014 was estimated as 2077 individuals, giving an average population density of 8.2 chamois/km^2 (Province of Lecco, unpublished data). The second area is in the Lepontine Alps, in the hunting district of Piedmont region (VCO2-Ossola Nord), with an extent of 72,740 ha (46°07' N, 8°17' E), ranging from 700 to 2400 m a.s.l. Here the chamois population was estimated as 1328 individuals in 2014, with an average density of 6.7 subjects/km^2 [18]. The third area is in central Italy, in the Abruzzo, Lazio and Molise National Park (ALMNP, 497 km^2, 41°44'N, 13°54'E), where samples were collected in Val di Rose, Mt. Meta, and Mt. Amaro subareas, ranging from 1650 to 2242 m a.s.l. In ALMNP, about 600 individuals of *R. p.ornata* were counted in 2014 [19], with local population densities of up to over 20 individuals/km^2 [20, 21].

Between August 2013 and January 2014, 103 fresh fecal samples were collected from *R. r. rupicapra* chamois harvested during the hunting season, whereas the 54 fecal samples from *R. p. ornata* were collected from the ground soon after defecation. To avoid the risk of collecting feces from the same individuals, sampling was carried out on different slope sites and took into account, as far as possible, the animals' sex and age. Fresh fecal specimens were collected and put into plastic bags, which were labeled and immediately packed in an insulated container with ice or cold packs. Specimens were then transported to the laboratory and processed within 1–3 days after collection.

Giardia *detection*

All 157 faecal samples were examined using an immunofluorescence (IF) test for the detection of *G. duodenalis* (Kit Merifluor® Meridian Diagnostic, Cincinnati, OH, USA).

The positive samples were frozen and subjected to Real-Time PCR for quantitative analysis (*q*PCR) using the *SSU-rDNA* gene, and to end-point PCR for genotyping using two genes i.e. *SSU-rDNA* and *gdh*.

DNA extraction

I.F. positive fecal samples were washed three times with PBS and subjected to 5 cycles of freezing with dry ice and thawing at 95 °C (5 min each step). DNA extraction was automatically performed by EZ1 BioRobot (Qiagen, Germany) following the manufacturer's instructions. To obtain a high quality DNA, samples were purified by Amicon Ultra-0.5 Centrifugal Filter Unit (Millipore) following the manufacturer's instructions.

*Quantitative (q*PCR*) and melting curve analysis*

A sequence of *G. duodenalis SSU-rDNA* gene (KJ888984) [22] was selected as reference target to design the plasmid control. The pEX-A vector (Eurofins, MWG/Operon, Ebersberg, Germany) was used to insert a fragment of approximately 293 bp of *G. duodenalis SSU-rDNA* gene.

The concentration of the pEX-A2 *G. duodenalis* plasmid was measured using a fluorometer, and the corresponding copy number was calculated using the following equation:

pEX-A2 *G. duodenalis* (copy numbers) = 6.02×10^{23} (copy/mol) × pEX-A2 *G. duodenalis* amount (0.31×10^{-5} g/ml)/pEX-A2 *G. duodenalis* length (293 bp + 2450 bp) × 660 (g/mol/bp) [23].

Ten-fold serial dilutions of the pEX-A2 *G. duodenalis* plasmid (from 1.03×10^{7} to 1.03×10^{-3} copies/μl) were used to assess the sensitivity, repeatability and reproducibility parameters of the assay, and to determine the quantity of the unknown samples based on linear regression calculations of the standard curve.

Amplifications and melting analysis were performed in the CFX-96 Real Time Instrument (BioRad, Italy). *G. duodenalis* ssRNA primers were GiarF (5'- GAC GCT CTC CCC AAG GAC-3') and GiarR (5'- CTG CGT CAC GCT GCT CG-3') [24].

The PCR mixture (final volume 20 μl) contained 1 μl of the plasmid-based control (or 5 μl of genomic DNA sample from 1 to 5 ng), 5X EvaGreen® Reagent (cat. No. 172–5201; BioRad, Italy) and 0.5 μM final concentration of each forward and reverse primer. Samples without genomic DNA (negative controls) were included in each PCR run. The cycling conditions in a CFX-96 thermocycler (BioRad) were as follows: initial denaturation at 98 °C for 2 min, followed by amplification for 35 cycles of 98 °C for 5 s and 55.6 °C for 15 s.

Fluorescence data were collected at the end of each cycle as a single acquisition. After amplification, the PCR products were melted by raising the temperature from 70 to 95 °C, with an increment of 0.5 °C/5 s, in order to denature and re-anneal before the high resolution melting; changes in fluorescence were recorded

with changes in temperature (d*F*/d*T*) and plotted against changes in temperature. The resolution melting curve (MC) profile was then analyzed using Precision Melt Analysis™ software version 1.2, with fluorescence (MC) normalization by selecting the linear region before and after the melting transition. The melting temperature (*Tm*) was interpolated from the normalized data as the temperature at 50 % fluorescence. Samples' melting curves were distinguished by plotting the fluorescence difference between normalized melting curves. *Tm* and standard deviation (*SD*) were recorded for each positive control.

Test-positive samples were identified on the basis of a single melting peak, which was consistent with that of the homologous plasmid control. The melting peak was 92.50 °C for *G. duodenalis SSU-rDNA*.

The copy number for each positive sample was calculated by relating the *Ct* mean value of each sample obtained in *q*PCR to a standard curve obtained from the respective plasmid control. Since the number of copies of the *SSU-rDNA* gene ranges from 60 to 130 in one *Giardia* nucleus [25], we considered an average of 95 copies in one nucleus and a total for 4 nuclei of 380 copies in one cyst. The number of cysts in each sample was calculated as the number of copies obtained in *q*PCR divided by 380 in 1 µl and then in 100 µl (since the volume of DNA after extraction is 100 µl). Finally, since the number of cysts in each sample was obtained in 200 mg of fecal sample, the results were transformed for cysts per gram (CPG) with the formula: number of cysts obtained in 200 mg of fecal sample X 5.

End-point PCR

A nested PCR was performed to amplify a 130 bp region from the *SSU-rDNA* gene, using the primers RH4 and RH11 for the first step, and the primers GiarR and GiarF in the second amplification round, as used for *q*PCR [24]. An additional analysis was carried out by using a semi-nested PCR to amplify a 432 bp fragment with the primers GDHeF and GDHiR in the primary reaction, and GDHiF and GDHiR in the secondary [26]. In all PCR reactions, positive (*Giardia* DNA) and negative (no template added) controls were added. All PCRs were carried out in a 25 µL volume containing 12.5 µL PCR master mix 2X (Promega), 5 µL template DNA, 0.6 mM of each primer and 0.1 mM BSA, 4 % dimethyl sulfoxide (DMSO), and were performed in a TProfessional Basic Thermocycler (Biometra GmbH, Göttingen, Germany). PCR products were visualized by electrophoresis on 1 % agarose gel stained by SYBR Safe DNA gel stain (Invitrogen). Amplicons were purified using the mi-PCR Purification Kit (Metabion GmbH). Both strands were sequenced by Bio-Fab Research s.r.l. (Rome, Italy). Sequences were edited with FinchTV 1.4 software (Geospiza, Inc, Seattle, WA, USA). To assign *Giardia* isolates to the correct assemblage, a comparison of the *SSU-rDNA* sequences by multiple alignments was performed using ClustalW2 software against known sequences available in GenBank. To test the significance of the results and to identify the sub-assemblage, a phylogenetic analysis was performed using MEGA6 software to compare the *SSU-rDNA* and *gdh* sequences with those of reference strains from different hosts. The best-fit model and parameters for tree construction were selected using the jModeltest software by the Akaike Information Criterion (AIC).

Results

G. duodenalis was detected by microscopy in seven out of the 157 fecal samples examined, (4.45 %; CI = 1.8–9.1). In *R. r. rupicapra* and *R. p. ornata*, a percentage of 5.82 % (6/103) (CI = 2.2–12.5) and 1.85 % (1/54) (CI = 0.5–9.8) were registered, respectively. All samples which tested positive to microscopy were also positive to *q*PCR and end-point PCR to one or both genes (i.e. *SSU-rDNA*, *gdh*). Overall, two assemblages i.e. A (with sub-assemblages AI and AIII) and E were identified (Table 1), and combined analysis of the two loci revealed no discrepancies in assemblage assignment.

At the *SSU-rDNA* locus, *G. duodenalis* assemblage A sequences (chamois no.s 10, 55, and 71) were identical to those from different hosts, including white-tailed deer in the USA (Genbank accession number KJ867494) previously reported by [27], dairy cattle (KF843922) in China [28] and Dutch patients (AY826206) [29] (Fig. 1). The isolates from chamois no.s 26, 32 and 93 matched with several assemblage E isolates from livestock (100 % similarity). These assignments were confirmed by the phylogenetic analysis as evidenced in Fig. 1.

Phylogenetic analysis of *gdh* sequences showed that isolate no. 71 from *R. r. rupicapra* clustered within sub-assemblage A/AI, sharing the same sequences as those from a large number of isolates from humans (L40509) [29, 30], several domestic animals, including cattle (EF507642) [31], and also from water (KM190761) [32], whereas isolate no. 10 from *R. p.ornata* clustered within the sub-assemblage AIII, together with a *Giardia* isolate from roe deer in the Netherlands (DQ100288) [29] and red deer in Poland (HM150751) [9] (Table 1; Fig. 2). Bootstrap analysis indicated strong statistical support for these grouping. PCR based on *gdh* locus failed for the other *Giardia* isolates. The nucleotide sequences obtained in this study have been deposited in EMBL/GenBank database under accession number from LN875379 to LN875384 for the *SSU-rDNA* gene and KT270858-KT270859 for the *gdh* gene.

The number of *Giardia* cysts in test-positive samples were predicted to range from 263 to 31,800 per gram of feces (Table 1).

Table 1 Number of individual chamois (*Rupicapra r. rupicapra* and *Rupicapra p. ornata*) investigated and test-positive to *Giardia duodenalis* by microscopy, *q*PCR and end-point PCR analysis

Collection sites	Species	Animal number	*Giardia duodenalis*			Assemblage/ Sub-assemblage	No. of CPG
			Microscopy	*q*PCR	PCR		
ALPS	*Rupicapra r. rupicapra*	1–3	–	–	–	–	–
		4	+	+	+	A	382
		5–25	–	–	–	–	–
		26	+	+	+	E	326
		27–31	–	–	–	–	–
		32	+	+	+	E	587
		33–54	–	–	–	–	–
		55	+	+	+	A	263
		56–70	–	–	–	–	–
		71	+	+	+	A/AI	31,800
		72–92	–	–	–	–	–
		93	+	+	+	E	11,200
		94–103	–	–	–	–	–
APENNINES	*Rupicapra p. ornata*	1–9	–	–	–	–	–
		10	+	+	+	A/AIII	618
		10–54	–	–	–	–	–

CPG cysts per gram of faeces calculated by *q*PCR

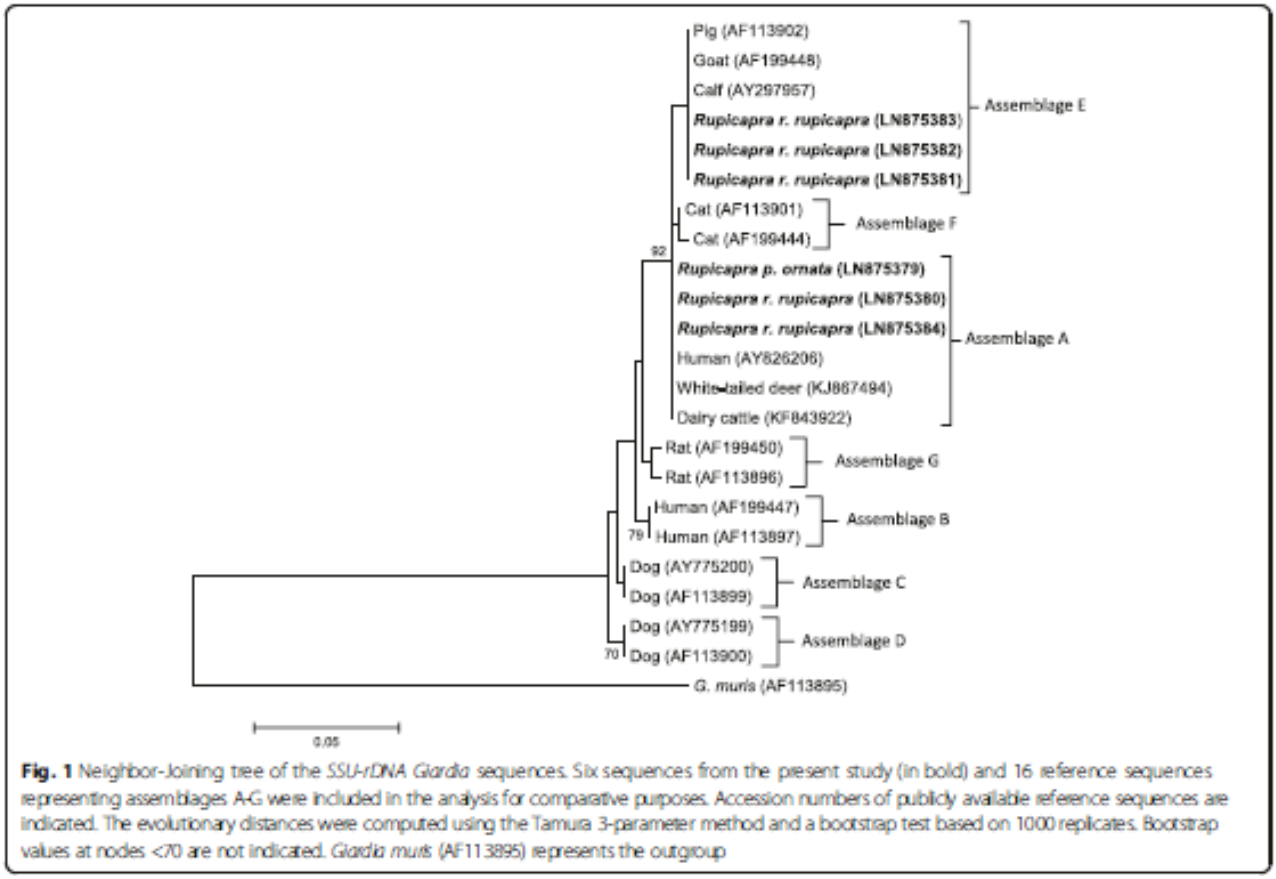

Fig. 1 Neighbor-Joining tree of the *SSU-rDNA Giardia* sequences. Six sequences from the present study (in bold) and 16 reference sequences representing assemblages A-G were included in the analysis for comparative purposes. Accession numbers of publicly available reference sequences are indicated. The evolutionary distances were computed using the Tamura 3-parameter method and a bootstrap test based on 1000 replicates. Bootstrap values at nodes <70 are not indicated. *Giardia muris* (AF113895) represents the outgroup

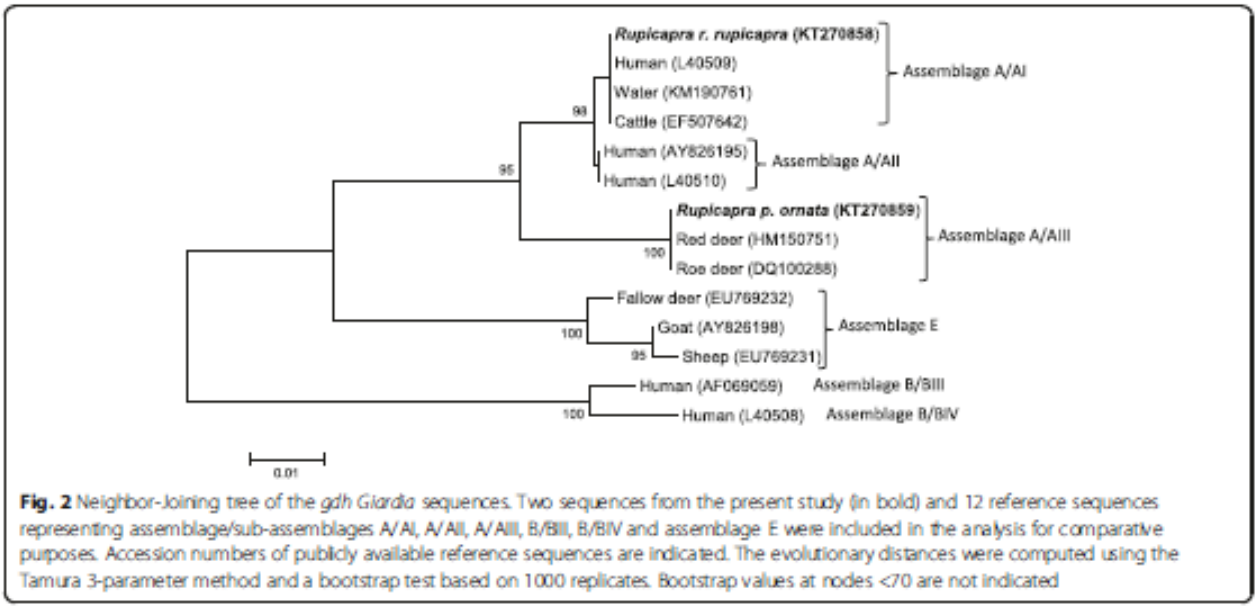

Fig. 2 Neighbor-Joining tree of the *gdh Giardia* sequences. Two sequences from the present study (in bold) and 12 reference sequences representing assemblage/sub-assemblages A/AI, A/AII, A/AIII, B/BIII, B/BIV and assemblage E were included in the analysis for comparative purposes. Accession numbers of publicly available reference sequences are indicated. The evolutionary distances were computed using the Tamura 3-parameter method and a bootstrap test based on 1000 replicates. Bootstrap values at nodes <70 are not indicated

Ethics

This research did not involve purposeful killing of animals. All fecal samples were gathered from dead free-ranging chamois legally shot by hunters in accordance with the Italian Law (157 of 11/02/1992) which implies that hunters have to carry culled wild ungulates to the control centres where, for each subject, age, sex, the shooting area and morpho-biometric measures are registered. Thus, no animals were killed specifically for this study.

Discussion

This is the first report of *G. duodenalis* in *Rupicapra* spp. Noticeably, the protist was found both in *R. r. rupicapra* and *R. p. ornata*, two different chamois subspecies living in quite distinct geographical areas, with an overall percentage value of 4.45 % (5.82 and 1.85 %, respectively), and a cyst burden of up to 31,800 cysts/g of feces. Assemblages A (AI and AIII) and E were detected.

Regarding the *G. duodenalis* genetic groups, it is known that assemblage A recognizes four sub-assemblages (AI, AII, AIII and AIV) [3]. Sub-assemblages AI and AII are found in both humans and animals; sub-assemblage AI – the zoonotic subtype – is preferentially found in livestock and pets, but has also been found in wild hoofed animals worldwide [6]; in Europe it has mostly been detected in cervids, i.e. fallow deer (*Dama dama*) in Italy [8], and red deer (*Cervus elaphus*) and roe deer (*Capreolus capreolus*) in Croatia [15]. Sub-assemblage AII is predominantly found in humans, whereas sub-assemblage AIII appears to be specifically associated with wild ungulates [6]; it has been isolated from cervids, i.e. red deer and roe deer in Croatia [15] and Poland [9], but also from cats [5, 33], and in a few cases from cattle [7]. AIV is almost exclusively found in domestic ungulates, and similarly to AIII it is only animal-related; therefore, both sub-assemblages are considered non-zoonotic [6].

Assemblage E is relatively host-specific, or rather 'group-specific', since it is limited to 'hoofed livestock' i.e. cattle, sheep, goats and pigs [6, 34–36], and for this reason it is known as the 'livestock genotype' [6]. However, assemblage E has been also detected in a wild hoofed cervid, i.e. fallow deer [33].

In the present study, the detection of assemblages A and E in chamois living in the Italian Alps and Apennines was not unexpected. It shows that also *R. r. rupicapra* and *R. p. ornata* chamois harbor assemblages/sub-assemblages A/AI/AIII, and confirms that assemblage E is associated to wild hoofed mammals, not only cervids [33] but also wild bovids, such as chamois *Rupicapra r. rupicapra*. In view of this, the term *'livestock genotype'* commonly used to classify genetic group E may be considered outdated and could possibly be replaced with the term *'hoofed animal genotype'*.

The presence of both assemblages A/AI and E in Alpine chamois can be related to their sharing of pastures with cattle and/or sheep and/or goats, as well as with cervids. In summer, farmers move their livestock up to high altitude alpine pastures, thus facilitating interaction with wild mountain ungulates [37]. Moreover, in addition to chamois, other species of wild ungulates are present in the alpine areas investigated; therefore, it is not only red deer and roe deer – cervid species found harbouring sub-assemblage AI *Giardia* in Croatia [15]- which may have an epidemiological role for *Giardia* trasmission, but also

alpine ibex (*Capra ibex*), a bovid species as yet uninvestigated for the presence of *Giardia*.

Furthermore, unlike other Alpine areas (i.e. the Dolomites), which attract thousands of human visitors involved in trekking and mountaineering, and where "tourist-borne" arrival of *Giardia* may be considered possible [14], the possibility of human-borne contamination by sub-assemblage AI appears unlikely in the *R. r. rupicapra* sampling areas, due to the remoteness of this territory accessible only to a few shepherds in summer and hunters in autumn. This seems to confirm that domestic and wild animals play a greater role in the dissemination of sub-assemblage AI than humans [6].

Conversely, in the Apennine area investigated where one positive chamois was detected (Val di Rose), the *R. p. ornata* population is totally isolated from domestic ruminants and never shares pastures with them [38]; more importantly, red deer and roe deer are present. Both species were reintroduced to ALMNP in 1972–1987 [39]; however, while red deer are present at high densities in the chamois range (with peaks of 0.5–1 deer/ha, in the grasslands of Val di Rose and Mt. Amaro), roe deer density is very limited, at least in summer [21, 38]. Based on this, and coupled with AIII detection in red deer and roe deer in Croatia [15] and in Poland [9], detection of wild ungulate-related sub-assemblage AIII [6] in the Apennine chamois can be fully justified.

Finally, although none of the investigated positive subjects showed signs of diarrhea, since only formed feces were collected, and the *Giardia* cyst burden was up to 31,800 cysts/g of feces, the pathogenic role of *Giardia* in wildlife remains unclear.

Conclusions

The findings of the present study indicate that *Rupicapra* spp. chamois harbor *G. duodenalis*. This is the first report of assemblage A/AI and assemblage E in *R. r. rupicapra* and AIII in *R. p. ornata*. The epidemiological roles that these wild bovids play in environmental contamination (including watercourses and watersheds) and transmission to other wild and domestic mammals or even humans, of zoonotic (A/AI) and/or non-zoonotic assemblages/sub-assemblages (E, AIII), require further investigation, as does the impact of *Giardia* on the health and sustainability of chamois populations, together with the possible cumulative effects of other pathogens [11].

Abbreviations

ALMNP: Abruzzo, Lazio and Molise National Park; qPCR: Quantitative real time PCR.

Competing interests

The authors declare that they have no conflict of interests.

Authors' contributions

SDA, AG and PL conceived the study. CDL performed the microscopy study. FB, MM, MS and LP carried out the molecular genetic studies. TT and FF collected samples. AG, CDL and FB drafted the paper, and all authors contributed significantly to editing the manuscript. All authors read and approved the final version of the manuscript.

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Chapter 3

Giardia and *Cryptosporidium*: epidemiological view on alpine wild ungulates

Manuscript

Abstract

Survival and spread strategy of *Giardia* and *Cryptosporidium* lie in their being multi-hosts pathogens infecting a wide range of hosts among humans and animals in quite different environments. Since little is known about the presence of these protozoa in alpine wildlife, above all respect to molecular perspective, a total of 561 faecal samples were collected from chamois (*Rupicapra r. Rupicapra*), red deer (*Cervus elaphus*) and roe deer (*Capreolus capreolus*) in Italian Alps. Immunoenzimatic analyses have been carried out through an ELISA commercial kit in order to spot surface copro-antigens of the protozoa. *Giardia* showed prevalences of 4,5% (11/246), 2,5% (5/196) and 8,4% (10/119), while *Cryptosporidium* of 1,2% (3/246), 0,5% (1/196) and 3,4% (4/119) respectively in chamois, red deer and roe deer. Among positive samples 14 have been subjected to nested PCR in order to define genotypes circulating and 8 were confirmed positive (4 chamois, 3 red deer and 1 roe deer respectively). Assemblage A of *Giardia* was detected in first two hosts species, suggesting a potential, although rather low zoonotic risk. Assemblage E was detected in chamois, highlighting a possible trasmission of this parasite between livestock and wildlife. Roe deer sample DNA sequencing failed. Concerning *Cryptosporidium*, immunoenzimatic positivity (n=8) haven't been confirmed by PCR.

Keywords: *Giardia*, *Cryptosporidium*, wild ungulates, Alps, ELISA, PCR

Introduction

Parasites are organisms that naturally developed different strategies of survival and spread in the environment. In particular some pathogens are frequently specialised, infecting only one or a few related host species, usually with limited impact (Malpica et al. 2006, Gortázar et al. 2007). Other parasites are multi-host pathogens infecting a wide range of subjects belonging to different taxonomic groups, performing direct or indirect lifecycle (Malpica et al. 2006). The multi-host pathogens are characterized by highest spread capacity, and they could be responsible for emerging infectious disease outbreaks in humans, livestock and wildlife, where they spatially overlap (Woolhouse 2002).

Giardia and *Cryptosporidium* belong to the latter parasite category. Different assemblages of *Giardia* (A and B) and genotypes of *Cryptosporidium* (mostly *C. parvum* and *C. hominis*) are recognised as zoonotic and have been isolated in livestock and wildlife (Appelbee et al. 2005, Smith et al. 2007). They are indeed infecting a wide range of hosts among humans, domestic and wild animals, therefore molecular characterization is needed to define their epidemiological role, also in relation to any interspecific interactions. In particular any zoonotic risk is favoured by oro-faecal cycle and survival of cysts and oocysts for weeks or even months in soil and water; faeces in fact contaminate streams, lakes and even urban water supply (Cacciò et al. 2005, ten Veldhuis et al. 2010). Furthermore, the infective dose is very low since a very small number of cysts and oocysts can cause infection (Skotarczak 2010). They can seriously affect immunocompromised subjects, especially with concomitant diseases, children and pregnant women (Nissapatorn and Sawangjaroen 2011).

While presence of these protozoa is well documented in livestock, with severe consequences mainly on juvenile classes and economic loss (Geurden et al. 2008, Santín and Fayer 2009), knowledge in wild ungulates isn't so wide, above all at molecular level. Few studies conducted in Norway, Great Britain and Spain recorded only prevalence of *Giardia* and *Cryptosporidium* among cervids and wild boar (*Sus scrofa*) (Sturdee et al. 1999, Hamnes et al. 2006, Robertson et al. 2007, Castro-Hermida et al. 2011, García-Presedo et al. 2013).

In Italian Alps wild ungulates could have a role in maintenance and spread of these protozoa in the environment, considering their impressive increase in the last decades (Carnevali et al. 2009) and shared rangelands with livestock during summer grazing with sanitary interaction (Gaffuri et al. 2006, Manfredi et al. 2007). At epidemiological level any zoonotic risk cannot be excluded. Moreover anthropic pressure due to traditional activities, like breeding and hunting, and a relevant intensification of outdoor sports throughout the year can cause disturbance, also considering the associated presence of dogs, even leading to stress state, allowing protozoa to have consequences on populations dynamics.

Therefore serological and molecular investigations were carried out in alpine chamois (*Rupicapra r. rupicapra*), red deer (*Cervus elaphus*) and roe deer (*Capreolus capreolus*) in Italian Alps to investigate (1) the spread and the epidemiology of these protozoa in the populations; (2) their molecular characterisation in order to evaluate the zoonotic and zoo-economic potential related to the infection.

Materials and Methods

Study area

The study area lies in the north easternmost part of Piedmont region, close to the border with Switzerland (Canton of Valais and Canton of Tessin), in the Lepontine Alps (Italy) (46°07'N, 8°17'E). The activity has been carried out in two contiguous territories: Alpe Devero that has a total extent of 5.454 ha within Alpe Veglia- Alpe devero Natural Park, having an altitude ranging from 1.600 to 3.552 m a.s.l., in which hunting is banned. The second area is hunting district of Verbano Cusio-Ossola province (VCO 2 - Ossola Nord) with an extension of 72.740 ha, ranging from 400 to 2400 m a.s.l.. Concerning wild ungulates populations in Alpe Devero , alpine chamois is the most represented with an estimated population size of 102 subjects (density of 1,87 subjects/kmq), 67 red deer (1,22 subject/kmq), but no data about roe deer are available. In hunting territory chamois population was estimated in 1.328 individuals (6,7 subjects/kmq), 574 red deer (2 subjects/kmq) and 655 roe deer (2.5 subjects/kmq) at summer 2014 census. Zootechnical activity is present throughout the study area, including the whole a population of 5.045 bovines, 9.117 sheep and 11.261 goats (www.izs.it 2014). During the whole year outdoor activities in study area are consistently present with over 3 million visitors in 2014 (www.piemonte-turismo.it).

The study area has been selected because in Alpe Devero the census time series in the last years showed a dramatic decline in chamois yearling, compared to the number of kids censused in the previous year, suggesting a summer mortality. Actually, considering that enteric protozoa can cause juvenile mortality, at least in livestock, an immunoenzimatic survey on faecal samples was carried out and *Cryptosporidium* has been found in two class 0 (Trogu 2012).

Sampling

A total of 561 faecal samples were collected during the biennium 2013-2014. In the Park animals were previously observed and localized through transects, then faecal samples were collected from ground respectively by 90 chamois, 79 red deer and 6 roe deer. As regard to hunting district, at the wildlife control centres where hunters have to carry culled ungulates in accordance with the Italian Law (157 of 11/02/1992) we took the opportunity for sampling faecal

specimens from rectal ampoule of 167 chamois, 106 red deer and 113 roe deer. Samples were split in two parts: one was stored at -20°C for immunoenzimatic analyses, the other one was conserved in Potassium dichromate solution (2,5%) for further molecular investigation.

Immunoenzimatic analyses

A commercial immunoenzimatic kit validated for human, with a sensibility and specificity of 96% and 100% respectively (RIDASCREEN® *Giardia*; RIDASCREEN® *Cryptosporidium*) and previously used for animal species such as dogs, cattle and cat (Itoh et al. 2005, 2006, Cardona et al. 2011, Helmy et al. 2013) has been used in according to manufacturer recommendations.

Molecular analyses

Genomic DNA was extracted from 200 mg of immunoenzimatic positive samples. After three cycles of sonication, QIAamp DNA Stool Mini Kit (Qiagen, Italy) was used according to the manufacturer recommendations, except for the 56°C overnight incubation after addition of Proteinase K.

A fragment of the SSU-rDNA of *Giardia* (130 bp) was amplified by a nested PCR protocol using RH11 forward 5'-CATCCGGTCGATCCTGCC-3' and RH4 reverse 5'-AGTCGAACCCTGATTCTCCGCCAGG-3' (Hopkins et al. 1997) in the first step and GiarF forward 5'-GACGCTCTCCCCAAGGAC-3' and GiarR reverse 5'-CTGCGTCACGCTGCTCG-3' in the second PCR (Read et al. 2002). PCR were performed in 25 µl containing 5 µl of DNA sample (De Liberato et al. 2015) in a thermocycler (Bio-Rad T100 Thermal Cycler) under the conditions of Hopkins et al., 1997. Amplification products (10 µl) were subjected to electrophoresis in 1% agarose gel stained with ethidium bromide.

A nested PCR protocol was used to amplify the 830 bp fragment of the SSU-rRNA gene of *Cryptosporidium* (Xiao et al. 1999, 2001, Li et al. 2015). The specific primers used were F1 forward 5'-TTCTAGAGCTAATACATGCG-3' and R1 reverse 5'-CCCATTTCCTTCGAAACAGGA-3' for primary PCR and F2 forward 5'-GGAAGGGTTGTATTTATTAGATAAAG-3' and R2 reverse 5'-AAGGAGTAAGGAACAACCTCCA-3' for the second. A second nested PCR protocol (Gatei et al. 2007) was performed to amplify the 800-850 bp fragment of the gp60 gene. The specific primers were GP60F forward 5'-ATAGTCTCCGCTGTATTC-3' and GP60R1 reverse 5'-GGAAGGAACGATGTATCT-3' for primary PCR and GP60F2 forward 5'-TCCGCTGTATTCTCAGCC-3' and GP60R2 reverse 5'-GCAGAGGAACCAGCATC-3' for secondary PCR.

Amplicons were purified using Wizard® SV Gel and PCR Clean-Up System (Promega) and DNA were sequenced by Primm Biotech (Milan, Italy). The sequences were aligned with those in GenBank to assign the genotype using the software MEGA6.

Statistical analyses

Through Generalized Linear Models (GLM) conducted by Software IBM SPSS version 19, dichotomous variable infected and non-infected related to *Cryptosporidium* and *Giardia* in VCO2 have been modelled to investigate the epidemiology of infections related to different explanatory variables (age, sex, year of sampling and altitude of culling localities) Any differences with p value <0,05 were considered statistically significant. Post-hoc analyses was performed when statistically significant factors were detected.

Results

Giardia showed prevalences of 4,5% (11/246), 2,5% (5/196) and 8,4% (10/119), while *Cryptosporidium* of 1,2% (3/246), 0,5% (1/196) and 3,4% (4/119) in chamois, red deer and roe deer respectively. Statistical models were significant only for roe deer that showed higher prevalences for both protozoa. In this host, concerning *Giardia* infection, models pointed out a significantly influence by age class with higher probability of calves being infected (p=24,1%) than adult (p=1,8%) (post-hoc test p=0,009). No significant differences were recorded between yearling (p=6,6%) and the other age classes (post-hoc test p>0,05), as well as between males and females. In 2014 probability of being positive is significantly higher than in 2013 (p=0,047). Finally animals culled at lower altitude (around 600 m. a.s.l) .have higher significantly probability to contract the infection (p=0,015) (Table 1).

		Coeff.	Wald Chi-Square	df	P value
(Intercept)		-1,026	0,647	1	0,421
Year			3.940	1	0.047
	2013	-1.662			
	2014	0			
Age class			7.979	2	0.019
	kids	3.178			
	yearlings	1.666			
	adults	0			
Shooting altitude			5.972	1	0.015

Table 1: Minimal model of factors influencing the probability for roe deer to be infected.

From *Giardia* immunoenzimatic analyses, 14 positive samples have been analysed by nested PCR, confirming 8 positivity among all three host species monitored (4 chamois, 3 red deer and 1 roe deer). Sequences obtained from amplicons were aligned with reference strains in GenBank (accession number: assemblage A (AF199446), assemblage C (AF199449), assemblage D (AF199443), assemblage E (AF199448) (Berrilli et al. 2004). Assemblage A was detected in both chamois and red deer, while assemblage E was detected only in chamois. Sequencing of roe deer purified DNA failed.

About *Cryptosporidium* all 8 immunoenzimatic positive samples were analysed by means of two nested PCR protocols and both resulted negative.

Discussion

Prevalences recorded for both protozoa in all 3 target host species are low, with values in favour of *Giardia*, in according to other investigations carried out in wild ungulates in different european contexts (Hamnes et al. 2006, Castro-Hermida et al. 2011), probably because of longer cysts emission (more than 30 weeks) compared to *Cryptosporidium* (1-2 weeks)(Olson et al. 2004).

Statistical analyses carried out on roe deer *Giardia* infection, confirm higher susceptibility of juvenile classes still building up immunitary system, at least as recorded in livestock, with severe economic loss (Noordeen et al. 2012, Rieux et al. 2013, Díaz et al. 2015). The higher probability of being positive in 2014 could be explained by frequent and intense rainfall occurred during summer, supported by higher average temperature that could have favoured cysts spread and survival (Mons et al. 2009). About shooting altitude, analyses show higher probability to get infection at lower ones. Even though during summer livestock move up in mountain pasture until middle September, all year long most human settlement and zootecnical actvities are localized down in valley. Here habitat is better for roe deer with consequent higher risk to get infection.and that could also explain the higher prevalences for both protozoa compared to chamois and red deer ranging higher altitudes with less anthropic pressure.

In red deer lower prevalences have been recorded for both *Giardia* and *Cryptosporidium*, with quite similar values between Alpe Devero and hunting district, suggesting a greater resilience to impact by humans activities; moreover chamois is the only one to be positive to both protozoa in both zones, probably because of wider sample size. The low prevalences and the limited sampling size about Alpe Devero (only 6 roe deer and 90 chamois), does not allow an evaluation concerning any differences between not-hunting area and hunting district.

In chamois assemblage A and E of *Giardia* were detected for the first time in agreement with a parallel study (De Liberato et al. 2015). Zoonotic assemblage A was isolated in red deer. This assemblage was already recorded in cervids

in Italy (Lalle et al. 2007) and other european countries (Beck et al. 2010, Solarczyk et al. 2012, García-Presedo et al. 2013), but as far as we know no data are available for Alps. The positivity of both host species has to be considered in relation to positive trend of red deer population and the high consistence of chamois in the Alps (Carnevali et al. 2009); and then favourable condition for the spread of this microrganism. Presence of assemblage E, known as livestock genotype and commonly detected in cattle, sheep and goats (Cacciò et al. 2005), indicates the close interactions between wildlife and livestock. Spreading of assemblage A suggests instead a zoonotic risk, although rather limited considering low prevalences recorded. No additional data are available about assemblage in roe deer because of the impossibility to sequence amplicons; quality of the DNA purified could be not optimal for sequencing, or it could lead to a false positive. Further analyses are ongoing to clarify these aspects.

Concerning *Cryptosporidium*, is necessary to test the real sensibility and specificity of the ELISA kit applied to wild ruminants; otherwise additional technique (immunofluorescence or/and microscopy) or PRC/qPCR could be performed to confirm any possible positivity.

Conclusions

This study shows the circulation of *Giardia* and *Cryptosporidium* in alpine wild ruminants; in particular with the detection in chamois of *Giardia* assemblage A and E, and in red deer of assemblage A. Only a previous investigation in Switzerland pointed out *Cryptosporidium* infection in free-ranging Alpine ibex (*Capra ibex ibex*) (Marreros et al. 2012). Overall prevalences recorded were very low in all three host species studied, however any underestimation related to sampling period can not be excluded. In effect critical point of these infections, for impact on the host and cysts/oocysts elimination, occurs during the first weeks of life, at least in domestic ruminants, like calves and kids (Trout et al. 2003, Manfredi 2010). Actually our sampling has been carried out during hunting season, occurring in autumn, when animals born in late spring are around 4/5 months old. On the other hand in Alpe Devero reaching areas used by females with few weeks old kids was impossible because of the snow still present until middle July. For these reasons, shifting of sampling, even of few weeks, could also cause an underestimated or unnoticed mortality among juvenile classes. About *Giardia* molecular characterisation, assemblage E detection highlights interactions between livestock and wild ruminants, likely during summer grazing. The finding of assemblage A in both chamois and red deer give evidence that different host species can be involved in its epidemiological role and this data has to be considered also in relation to human infection. Regarding the study area, at the moment zoonotic risk from wild ungulates doesn't appear severe, considering the low prevalences recorded. However it can not be underestimated taking into account the increase of

these populations and the intensification of human outdoor activity in alpine context. Further analyses are necessary to deepen knowledge about real *Cryptosporidium* circulation in alpine context.

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Chapter 4

Eimeria, epidemiological approach in alpine wild ungulates

Manuscript

Abstract

Eimeria is involved in different ruminant hosts and has often been considered as a partial limiting factor of wildlife populations affecting animal welfare. Despite the wide knowledge about coccidiosis in livestock, information about wild ruminants must be deepened to define *Eimeria* epidemiological role. A total of 414 faecal samples of wild ruminants (*Rupicapra r. rupicapra, Cervus elaphus* and *Capreolus capreolus*) were collected in Lepontine Alps, within Natural Park Alpe Veglia – Alpe Devero and in the contiguous hunting territory of VCO2. Faeces were analysed through FLOTAC methodology in order to quantify emissions of coccidian oocysts (OPG) and gastro-intestinal nematode eggs (EPG). *Eimeria* shows prevalence of 81,1% (137/169) in chamois, of 46,1% (78/169) in red deer and of 43,4% (33/76) in roe deer. Overall prevalences for gastro-intestinal nematodes are 76,4% (129/169) in chamois, 57,4% (97/169) in red deer and 67,1% (51/76) in roe deer. Statistical analyses were carried out to define the epidemiological role of coccidian infections in wild ungulates in Alpine context. Data recorded don't suggest any impact on population dynamics, and the need arises to deepen co-infections effects.

Keywords: *Eimeria*,OPG, gastro-intestinal nematodes, EPG, wild ungulates

Introduction

Parasites have often been considered as a partial limiting factor of wildlife populations affecting animal welfare. In particular little information is available on detrimental effects of *Eimeria* on wild ungulate population dynamics, considering that host characteristics, population density, seasonal variability (temperature and humidity) and individual predisposition could influence possible coccidiosis (Stancampiano et al. 2003).

Several species of *Eimeria* are involved in different ruminant hosts, but there is no cross infection due to the strict host specificity. In livestock coccidiosis is of great economic importance because of the losses derived by clinical diseases with characteristic diarrhoea and possible mortality, and subclinical infections characterised by severe weight loss (Andrews 2013). Clinical coccidiosis usually occur after massive ingestion of sporulated oocysts or during asexual multiplication in the host, in relation to a lowered resistance of the animal (Chartier and Paraud 2012).

Prevalence and intensity of *Eimeria* shedding are the highest in young animals of less than 4-6 months, at least in small domestic ruminants. The peak of excretion arise around weaning period, emission starts very early at 2–3 weeks of age following ingestion of sporulated oocysts during the first few days of life (Mundt et al. 2005, Tafti and Mansourian 2008).

Considering that sporulated oocysts show a great resistance in the environment, surviving several months or even more than a year (Chartier and Paraud 2012), it's interesting to study diffusion and epidemiology of these protozoa in mountain context, where cold weather and high umidity favour oocyst survival (Svensson 1994).

Previous surveys have been carried out about eimeriosis of wild ungulates in Italy and other european countries, descriptive investigations that underline prevalence data and morfological identification. About chamois (*Rupicapra rupicapra rupicapra*), study carried out in Italy (province of Belluno) showed high prevalence, ranging from 90% to 100% (Stancampiano et al. 2002). In red deer (*Cervus elaphus*) *Eimeria* prevalence is of 75% approximately (Pilarczyk et al. 2005, Kowal et al. 2015) , while in roe deer (*Capreolus capreolus*) about 52% (Poglayen et al. 1990, Pilarczyk et al. 2005). Aim of the study is to deepen the epidemiological significance of *Eimeria* in wild alpine ruminants (*Rupicapra r. rupicapra*, *Cervus elaphus* and *Capreolus capreolus*) assuming coccidia as useful indicators of immunological status in relation to health ecosystem. Actually in the last decades Italian Alps have suffered a dramatic land use change in relation to traditional activities strong contraction and, on the other hand, to increase of tourism and outdoor activities even more differentiated with direct and/or indirect human presence on territory all year long. That can induce a state of stress, interfering at immunological level, allowing possible disease development. Despite the host-specificity of these protozoa, and since wild ruminants often harbour other parasites, in particular gastro-intestinal nematodes (Bye 1987, Zaffaroni et al. 2000, Stien et al. 2002), possibility of coinfection with other micro and/or

macroparasites due to interaction with livestock, must be taken into account with respect to worsening of the pathogenic effect of coccidia (Taylor 2009).

The importance of studying trend of these parasitosis also lie in lack of evident symptoms in wildlife (Stancampiano et al. 2003). In particular, at least in small domestic ruminants, coccidiosis can be characterised by sudden mortality mainly amongst young animals 2 to 4 months old, usually they occur subclinically with weight loss, dehydration and worsening of animal general condition (Chartier and Paraud 2012).

Materials and Methods

Study area

The activity has been carried out in two contiguous areas with different management: Alpe Devero whitin Alpe Veglia-Alpe Devero Natural Park, where huntig is banned, and Hunting district of Verbano Cusio-Ossola province (VCO 2). Alpe Devero has an extension of 5.454 ha and an altitude ranging from 1600 to 3552 m a.s.l.. Among the species studied, chamois has a density of 1,87 subjects/kmq, and red deer of 1,22 subject/kmq. No data about roe deer are available. VCO2 has an extent of 72.740 ha; altitude range from 400 m a.s.l. to 2400 m a.s.l.. Among wild ungulates chamois represent the most abundand population in the area with 6,7 subjects/kmq, red deer with a population size of 2 subjects/kmq, roe deer of 2.5 subjects/kmq. These species are censused through annual censuses and are subjected to hunting selection.

In both areas zootecnical activity is quite developed having about of 5.000 bovines, 9.000 sheep and 11.000 goats (2014, BDN Anagrafe Zootecnica Ministero della Salute, CSN "G. Caporale" Teramo – www.izs.it); from May to October livestock usually move up to high pasture. Moreover different sport and recreational activities are carried out during the whole year, implying a relevant human presence on the territory.

Sampling

In biennium 2013-2014, within no-hunting area territory, transects with fixed observation points have been established. During transects, carried out either in early morning and in the evening, monthly from June to November animals have been surveyed by 10X50 binoculars, and by 60X telescope, and sorted as far as possible, by species, age and gender. Moreover we paid attention to any behavior or physical changes, e.g. body score condition, fur quality and correct molt, monitoring animals consistency to observe population trend and to exclude any decrement. Then the collection activity have been performed by picking only fresh samples of faecal material, reaching the animals sighting site. In all 175 samples were collected 77 from chamois, 89 from red deer and 6 from roe deer.

Regarding VCO2 we gathered faecal samples from rectal ampoule (92 chamois, 80 red deer and 70 roe deer).at the wildlife control center where hunters have to carry culled wild ungulates for registration of age, sex, shooting area and morpho-biometric data in accordance with the Italian Law (157 of 11/02/1992). Faecal samples were stored in Potassium dichromate solution (2,5%) for coprological analyses.

Coprological analyses

Eimeria oocyst per gram of faeces (OPG) and gastro-intestinal nematode eggs per gram of faeces (EPG) have been detected by FLOTAC methodology for higher sensibility (Cringoli et al. 2010), using Magnesium sulfate (FS6: MgSO4 - density 1,28) and Sodium nitrate (FS4: NaNO3 – density 1,20) respectively, to acquire quantitative and qualitative data about oocystis and eggs emission.

Statistical analyses

Statistical analyses are conducted by Software IBM SPSS version 19. Prevalence of *Eimeria* was calculated for each host species. Through Generalized Linear Models (GLM), oocyst emissions have been analized in realation to different explanatory variables. Any differences with p value < 0,05 were considered statistically significant.

Oocyst emissions have been modelled with negative binomial distribution through three statistical models for each host species to provide for the different data available about the two zones. The first model aims to compare Alpe Devero and VCO2 using communal variables: age class (kids and adultl), year and zone of sampling, as categorical variables and gastro-intestinal nematode eggs per gram of faeces (continuous). In this model we have excluded yearling class to make both areas surveyed population uniform. The second model, about VCO2, added variables such as sex (categorical) and altitude of culling (continuous). The last model, restricted to Alpe Devero, added variables such as months (categorical) and collecting district (categorical).

Results

Overall prevalences for *Eimeria* and gastro-intestinal nematodes are reported in table 1, and relatives value emissions in table 2.

		Alpe Devero	VCO2	Total
Chamois	***Eimeria***	59,7% (46/77)	98,9% (91/92)	81,1% (137/169)
	Gastro-intestinal nematodes	62,6% (49/77)	86,9% (80/92)	76,4% (129/169)
Red deer	***Eimeria***	48,3% (43/89)	43,7% (35/80)	46,1% (78/169)
	Gastro-intestinal nematodes	58,4% (52/89)	56,2% (45/80)	57,4% (97/169)
Roe deer	***Eimeria***	83,0% (5/6)	40,0% (28/70)	43,4% (33/76)
	Gastro-intestinal nematodes	33,3% (2/6)	70,0% (49/70)	67,1% (51/76)

Table 2: Prevalences of *Eimeria* and gastro-intestinal nematodes in target host species in Alpe Devero and VCO2.

		OPG *Eimeria*		EPG gastro-intestinal nematodes	
		Alpe Devero	VCO2	Alpe Devero	VCO2
Chamois	**Min**	0	0	0	0
	Max	2696	9232	588	1080
	Mean	282,45	1419,07	23,76	109,69
	Median	12	614	4	36
Red deer	**Min**	0	0	0	0
	Max	1320	638	386	52
	Mean	90,85	31,77	17,83	5,45
	Median	0	0	4	2
Roe deer	**Min**	0	0	0	0
	Max	1880	1192	8	84
	Mean	501,33	60,88	1,66	10,02
	Median	56	0	0	4

Table 3: Emission values of Eimeria and gastro-intestinal nematodes in target host species.

In all 3 host species studied juvenile classes demonstrate level of shedding significantly higher than adult classes ($p<0,05$) either in Alpe Devero and in Hunting district (Table 3, 4, 5).

Regarding analyses on different host species, chamois shed higher number of oocyst in July. No significant differences have been recorded with June (post-hoc $p=0,297$), but in September and in October emissions are significantly lower (post-hoc $p=0,000$ for both). *Eimeria* shows prevalences of 59,7% and 98,9% in Alpe Devero and in VCO2 respectively, moreover in this latter area oocysts emission strength is significantly higher than in Alpe Devero ($p=0,000$) (Table 3). Concerning co-infection *Eimeria* and gastrointestinal nematodes, no significant effect has been observed between OPG and EPG.

Chamois			Coeff.	Wald Chi-Square	df	P value
I MODEL Comparision areas	(Intercept)		6,831	2621,196	1	0,000
	Area			119,194	1	0,000
		Alpe Devero	-1,962			
		VCO2	0			
	Age class			47,067	1	0.000
		kids	1,499			
		adults	0			
II MODEL VCO2	(Intercept)		6,869	2262,578	1	0,000
	Age class			14,015	2	0,001
		kids	1,339			
		yearlings	0,553			
		adults	0			
III MODEL Alpe Devero	(Intercept)		2,527	19,066	1	0,000
	Age class			20,202	1	0,000
		kids	1,775			
		adults	0			
	Months			112,072	3	0,000
		June	1,076			
		July	2,271			
		September	-1,590			
		October	0			

Table 4: Minimal models of factors influencing coccidian emission in chamois.

Red deer emissions in 2013 were higher than 2014 (p=0,000) and number of oocysts shedding was higher in June (p=0,006); a significant difference between females and males has been recorded with higher shedding in the latter class (p=0,000). Considering EPG values, the increase of eggs output has useful effect on oocysts emission (p=0,002) (Table 4).

Red deer			Coeff.	Wald Chi-Square	df	P value
I MODEL Comparision areas	(Intercept)		1,876	98,363	1	0,000
	Year			24,122	1	0,000
		2013	0,942			
		2014	0			
	Age class			135,962	1	0.000
		kids	2,299			
		adults	0			
	Nematodes Eggs			97,705	1	0,000
II MODEL VCO2	(Intercept)		3,197	208,035	1	0,000
	Age class			65,914	2	0,000
		kids	1,656			
		yearlings	-1,416			
		adults	0			
	Sex			12,849	1	0,000
		female	-0,975			
		male	0			
	Nematodes Eggs			10,073	1	0,002

Red deer			Coeff.	Wald Chi-Square	df	P value
III MODEL Alpe Devero	(Intercept)		1,157	4,411	1	0,036
	Age class			45,836	1	0,000
		kids	2,050			
		adults	0			
	Year			16,432	1	0,000
		2013	1,583			
		2014	0			
	Months			80,254	4	0,000
		June	1,355			
		July	0,539			
		September	-1,794			
		October	-0,020			
		November	0			
	Nematodes Eggs			53,138	1	0,002

Table 5: Minimal models of factors influencing coccidian emission in red deer.

Concerning roe deer oocysts emission intensity is significantly higher in Alpe Devero than in VCO2 (p=0,000); about sex classes the same result related to red deer has been recorded (p=0,000), whereas increase of gastro-intestinal parasite eggs has a negative effect on oocysts shedding (p=0,003) (Table 5).

Roe deer			Coeff.	Wald Chi-Square	df	P value
I MODEL Comparision areas	(Intercept)		3,835	375,827	1	0,000
	Area			17,026	1	0,000
		Alpe Devero	1,836			
		VCO2	0			
	Age class			10,923	1	0.001
		kids	1,124			
		adults	0			
	Nematodes Eggs			8,950	1	0,003
II MODEL VCO2	(Intercept)		4,093	346,121	1	0,000
	Age class			72,413	2	0,000
		kids	3,239			
		yearlings	2,567			
		adults	0			
	Sex			48,077	1	0,000
		female	-2,475			
		male	0			

Table 6: Minimal models of factors influencing coccidian emission in roe deer.

Discussion

Eimeria prevalences are comparable with previous surveys carried out in chamois in Italian Alps (Restani 1968, (Stancampiano et al. 2002) and roe deer (Mantovani et al. 1970; Poglayen et al. 1990). In this study chamois have higher prevalences and intensity of oocysts emissions. Red deer and roe deer have lower consistency with resulting lower coccidia prevalences. Actually different host population size suggests an effect related to density. Higher density of chamois favours transmission and diffusion of enteric protozoa through animals direct or indirect contact on pastures (Stancampiano et al. 2003).

Comparing Alpe Devero and VC02 data, chamois shows significant higher OPG in hunting district. These areas appear similar at topographic level and at agro-zootechnical land management too, but hunting makes a relevant difference. This result could be expression of environmental stress due to hunting activity (a bag of more than 500 heads among chamois, red deer and roe deer are culled in each hunting season by around 900 hunters admitted in VCO2 – www.regione.piemonte.it/agri). Although hunting is banned in the Alpe Devero stress could arise by consistent outdoor activities, but access to chamois ranges by recreationists is strongly limited. Regarding roe deer this host show higher oocyst emissions within the Park. This result, assessed by statistical model, should be deepened because of unbalanced sampling (6 in Alpe Devero *vs* 47 in VCO2 – excluding yearling class), suggesting an environmental situation less favourable for this species. In effect its range in Alpe Devero overlaps with the most utilized areas for recreational activities also considering the growing presence of dogs; moreover the habitat with an altitude of over 1600 m a.s.l. is unsuitable for roe deer biology. Red deer prevalence for either *Eimeria* and gastrointestinal nematodes doesn't highlights any difference between the two areas and that could suggest a good adaptability to quite different environmental contexts.

Focusing attention on Alpe Devero, not considering information about roe deer because of statistical data lack, higher *Eimeria* emissions in chamois and red deer were detected in summer months (June and July), following birth months (May – June) when females are in lactation and metabolic cost could affect immunitary system, favouring parasitosis susceptibility. Moreover younger animals are more susceptible to protozoan infections as well known in livestock (Lassen et al. 2009, Chartier and Paraud 2012).

Analyzing data from VCO2 about sex classes it's apparent that males of both red deer and roe deer have higher emissions than females. Hunting season persists from middle September to late November, partially overlapping to the red deer rutting season. Considering that reproductive success is expected to result from competition between dominant males (Stopher et al. 2011) this behaviour causes a great strength waste in the animals, resulting in possible

immunosuppression. That could happen also for roe deer because of its strong territoriality from early spring to late summer during mating season, just before hunting, and then animals are in the process of energy recovery.

Considering the simultaneous emissions of oocysts and gastro-intestinal nematode eggs in both areas, the 3 host species show different pattern: in chamois it arises that EPG doesn't influence OPG; in red deer a susceptibility to coinfections has been recorded with concomitant increase of both values, while in roe deer it appears that egg emission inhibits *Eimeria* shedding. The result in roe deer has to be considered in relation to unbalanced sampling, considering that in Alpe Devero animals with highest oocysts emissions, were negative for eggs of gastro-intestinal nematodes.

Since micro and macroparasites are integral components of the ecological communities that include their hosts (Pedersen and Fenton 2007), the interspecific interactions could explain communities response to external events, however their role in influencing parasite dynamics is unclear (Lello et al. 2004), in fact some degrees of interaction, sometimes very dramatic, could occur (Cox 2001). Since coinfection of multiple parasite species represents the rule rather the exception (Knowles et al. 2013), we need to deepen this matter even more in wildlife contexts.

In this perspective the achievement of a consistent data series about infections is a basic step also in relation to evaluate any impact on free-ranging populations dynamics. Present results, compared to data census available for target host species in the area, don't suggest an impact of these protozoa.

Conclusions

This study highlights the complexity of multifaceted Alpine context in relation to different factors that interact on parasite- host-environment association, with intrinsic implications on wildlife populations dynamics. This approach is useful in order to define management strategies in a health ecosystem perspective, even more when option is opposite, e.g. hunting or no-hunting. In this context welfare of free-ranging animals represents a basic indicator for environmental quality, and their parasites are themselves ecological markers as confirmed in the present study. Actually not always field conditions allow to have a complete framework of biotic and abiotic factors to achieve the expected aims, beginning from a consistent sample size. Moreover ethic reasons can arise in relation to any perturbation studies on parasite community, e.g. anthelminthic treatment.

Considering that numerous host species share their ranges, in particular domestic and wild ruminants during summer pastures, and the real possibility of parasite cross infection, *Eimeria* represents a useful model, more than gastro-intestinal nematodes, to evaluate parasite host interaction because of its strict host-specificity.

On the other hand gastro-intestinal nematodes or other pathogens could affect *Eimeria* infections and then the need to deepen our knowledge about co-infections is confirmed, and it is desiderable in wildlife not only for scientific reasons but for management implications.

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Chapter 5

Conclusions

The aim of this study was to deepen the knowledge about presence of three common intestinal protozoa, *Giardia*, *Cryptosporidium* and *Eimeria* in wild ungulates, in order to define the epidemiological value of these parasites in wildlife population and at the same time to understand the involvement of wild animals in maintenance and spread of protozoa in the environment, related to possible zoonotic, economic and conservationist implications.

5.1 Summary of the main results

G. duodenalis has been detected, through immunofluorescence, for the first time in Alpine and Apennine chamois with prevalences of 5,82% and 1,85% respectively (see **Chapter 2**). All positive samples were confirmed by *q* PCR and end-point PCR, highlighting presence of assemblage A/AI and E in *R. r. rupicapra*, and presence of assemblage AIII in *R. p. ornata*. These results suggest a circulation in alpine context of zoonotic assemblage and it could be an issue for public health; instead, identification of assemblage E, typical of livestock, highlights interaction between chamois and domestic animals. Assemblage AIII is characteristic of wild ungulates, considering that in the Apennine sampling areas chamois are rarely overlapped with livestock, this result was expected.

A parallel survey has been carried out through immunoenzimatic analyses (see **Chapter 3**). *Giardia* showed prevalence of 4,5% (11/246), 2,5% (5/196) and 8,4% (10/119) while *Cryptosporidium* of 1,2% (3/246), 0,5% (1/196) and 3,4% (4/119) respectively in chamois, red deer and roe deer. Statistical analyses carried out in roe deer show higher probability of calves being infected than adult; in 2014 probability of being positive has been significantly higher than in 2013. At last animals culled at low altitude have higher significant probability to contract the infection. Molecular investigations highlight presence of assemblage A and E in chamois, in agreement with De Liberato et al. (2015), and assemblage A in red deer. In alpine context these results demonstrate the potential zoonotic risk due to the increasing relation between humans, wildlife and livestock. Concerning *Cryptosporidium* contrasting results between immunoenzimatic and molecular analyses highlight the need of further analyses to exclude any false positive samples and/or because of DNA quality .

Faecal samples of the same animals were analysed through copromicroscopic analyses to investigate presence of intestinal coccidia and gastro-intestinal nematodes (see **Chapter 4**). *Eimeria* shows overall prevalence of 81,1% in chamois, 46,1% in red deer and of 43,4% in roe deer; juvenile classes demonstrate significantly higher level of shedding than adult classes. Chamois have higher prevalence and intensity of oocysts emissions compared to other hosts, showing higher shed in hunting territory and in summer. In red deer and roe deer different emission between sex classes is interesting, showing higher oocysts shedding in males, probably because of their stressful mating period. Overall prevalences for gastro-intestinal nematodes are 76,4% in chamois, 57,4% in red deer and 67,1% in roe deer. The

increase of eggs output has an useful effect on oocysts emission in red deer, while has a negative effect in roe deer. The latter results has to be deepened because of unbalanced sampling (in roe deer with the highest oocysts emission, eggs output has not been recorded).

5.2 Concluding remarks

Considering data about protozoal infection in literature, we can observe a wide documentation about *Giardia*, *Cryptosporidium* and *Eimeria* detection in livestock, but in wildlife only prevalence data are available for some species. Only few information about molecular analyses of these protozoa in species as chamois, red deer and roe deer are available. In alpine wild ruminants; only a previous investigation in Switzerland pointed out *Cryptosporidium* infection at morphological level, without molecular characterization in free-ranging Alpine ibex (*Capra ibex ibex*) (Marreros et al. 2012)

Concerning *Giardia* and *Cryptosporidium*, the low overall prevalences recorded in both protozoa highlight, in all 3 target species, *Giardia* higher widespread, also reported in wild animals in other european contexts (Hamnes et al. 2006, Castro-Hermida et al. 2011). The low prevalences could be understimated in relation to sampling period. Indeed, considering hunting season (October-November) and sampling difficulties in Alpe Devero due to snow still present at low altitude until summer months, we were not able to sample during the neonatal period, critical point for these infections (Trout et al. 2003, Manfredi 2010). Assemblage A was already recorded in cervids in Italy (Lalle et al. 2007) and other European countries (Beck et al. 2010, Solarczyk et al. 2012, García-Presedo et al. 2013), while data about chamois are new. At the moment zoonotic risk related to wild ungulates does not appear severe, considering the low prevalences recorded. However it cannot be underestimated taking into account the increase of these populations (Carnevali et al. 2009) and the rising outdoor activities. Assemblage E detection, known as livestock genotype and commonly detected in cattle, sheep and goats (Cacciò et al. 2005), highlights interactions between livestock and wild ruminants. Further analyses are necessary to deepen knowledge about real *Cryptosporidium* circulation in alpine context. Additional technique (immunofluorescence or/and microscopy) or PRC/qPCR could be performed to confirm any possible positivity.

This study also confirms presence of *Eimeria* in alpine context among all species studied, with prevalences in agreement with previous survey (Poglayen et al. 1990, Stancampiano et al. 2002). Data recorded in this study are comparable to knowledge about livestock, above all in regard to higher susceptibility of juvenile classes that were still building up immunitary system (Noordeen et al. 2012, Rieux et al. 2013, Díaz et al. 2015).

The complexity of the subject has to be considered also in relation to any co-infection with other micro and/or macroparasites. We can't state anything about their impact on populations: a data series about parasitosis in wild ungulates is desiderable to deepen the role of intra and interspecific interactions, and host response to parasite infections. For this latter purpose *Eimeria* can be a useful model to better understand parasite-host-environment relation.at intraspecic level.

Thanks to hunting district and to Natural Park Alpe Devero, it has been possible to compare data census available for target host species with all data recorded.

Concerning Alpe Devero chamois and red deer population have slight negative trend since 1993, however chamois kids decrease occurred in summer 2011 appear as a circumscribable event. The low prevalence recorded for *Giardia* and *Cryptosporidium*, and the moderate intensity of *Eimeria* oocysts emissions don't suggest any impact of these protozoa on population dynamics. Therefore population reduction should be investigated in relation to other extrinsic or intrinsic factors. On the other hand in VCO2 chamois and red deer show a total positive trend (since 2004), and only roe deer population decreased in these years. Although this host shows the highest prevalences for *Giardia* and *Cryptosporidium*, values are low and doesn't appear to be able to explain that population decrement.

Research about wildlife isn't always easy. In general, wildlife diseases surveillance should be based on passive surveillance and direct test to ensure the detection of a pathogen as soon as it is introduced. Active surveillance might play an important role in supporting the passive one, in case of inadequacy of passive surveillance or of limited sample size (Guberti et al. 2014). In our studies collaboration with hunting activity was determinant and allowed having basic information about culled animals, such as age, sex, morph biometric measures and observation of possible lesions not observable in live free-ranging animals. However survey is strictly related to hunting season during autumn, showing only a limited window in wildlife diseases ecology. On the other hand, although not-invasive sampling in natural park doesn't allow to collect information on individual animals, it can give more data concerning host population dynamics and parasite trends throughout the year, besides logistical difficulties and weather conditions.

Concluding, data recorded give new information about protozoa presence in alpine wild ungulates, in particular with respect to the role of wildlife in potential zoonotic risk, at least for *Giardia* infections. Further analyses are necessary to define presence and epidemiological role of *Cryptosporidium*. Concerning *Eimeria*, even if is not a new record in alpine ruminants, data collected in this study allow to clarify its epidemiology in relation to intrinsic and extrinsic factors, as individual characteristics and presence of different human activities on the territory respectively.

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