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WILD GAME MEAT: HEALTH MONITORING TO PROTECT CONSUMERS

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Introduction

Wildlife has gained an increasingly importance worldwide during last decades not only for its unquestionable biological and environmental significances but mainly for its central epidemiological role in transmission of infections. Indeed free-ranging populations can contribute to the circulation of many important zoonosic diseases (Henaux et al., 2013; Michel et al., 2013), considering besides that more than 70% of emerging zoonoses are hosted in wild animals (Kruse et al., 2004; Kuiken et al., 2005; Jones et al., 2008), of zooeconomic pathogens (Garcia-Jimenez et al, 2013; Rhyan et al., 2013) and of infections that can threaten species conservation (Thorne and Williams, 1988; Rahman et al., 2010). Moreover these considerations must be seen also in relation to the growing of human driven land-use change, which frequently includes encroachment into wildlife habitat (Miller et al., 2013), the demographic rise of free-ranging populations that promotes an increase of the potential contact and pathogen transmission at the livestock-wildlife interface (Miller et al., 2013) and climate change. Therefore it emerges the importance to provide a constant health monitoring of wild animals to give evidence about the circulation of diseases and to manage their spread within populations. Although many of these infections appear in the OIE list (http://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2012/), there is a lack of agreement about systematic health surveillance programs (Kuiken et al., 2005), usually defined just in crisis, which are often not well integrated with human and domestic animals health monitoring. Besides, this deficiency of a regular health control of wildlife takes on still more importance in relation to the consumption of game meat products. Although game meat had served as food for such a long time and it had not been a main concern of food hygienists for quite a long time (Paulsen, 2011), the new European food hygiene regulations (Reg. EC. 852, 853, 854/2004) has legislated about the auto consumption and the commercialization of game meat, according to specific requirements, making hunters primary producers. Indeed these regulations have promoted a real change in the spread of game products since traditionally they were used for domestic consumption or for preparations relocated to local trade, but now their distribution is increasing on a large scale. Moreover once the risk of food-borne infections was remarkably lower because game meat was usually cooked for a long time but nowadays people tend to eat raw or under cooked meat, such as fresh sausages or *roast beef* or *carpaccio* (thinly sliced raw meat). Consequently a proper exploitation (also from an economic viewpoint) of game bags would be appropriate not only for its "productive" value but also for its significant market price intrinsic to the organoleptic quality of the carcasses and typicality of products (Winkelmayer, 2010). However quality and hygiene assurance of game meat are strictly related to few critical steps that must be respected to guarantee a fit product for human consumption. On one hand undesirable pain or

suffering before the hunting or handling procedures (capture, restraint) should be avoided to prevent adverse effects on meat quality (Casoli et al., 2005). On the other hand the circumstances of shooting can influence muscle contamination by microorganisms from the hide or the gastro-intestinal tract or in the muscle tissue itself (Gill, 2007), a delay or incorrect bleeding has a negative effects on the hygienic and organoleptic quality and the shelf-life of meat (Casoli et al., 2005) or during the skinning, the operator must adopt particular attention not to contaminate the surface of the carcass and evisceration must begin immediately after the killing and must be completed in the minimum time possible (Casoli et al., 2005; Gill, 2007; Giaccone, 2010). Then the quick refrigeration of carcasses prevents the development of microflora ensuring the handling and transportation processes which may affect the intrinsic biochemical qualities of meat, especially in warm months (Paulsen and Winkelmayer, 2004). In particular, these practices should be applied to avoid the microbiological contamination with *Enterobacteriaceae* (*Salmonella* spp. or *Yersinia enterocolitica*) or Coliform bacteria (*Escherichia coli* and Total coliform bacteria) or an elevated Total Bacterial Load (TBL) that can provoke food-borne diseases, serious sometimes, in consumers.

These considerations must be evaluated also in relation to our Country. Although during last decades several surveys demonstrated the circulation of different-value pathogens in Italian free-ranging populations (Ferroglio et al., 1998; Ferroglio et al., 2000; Dini et al., 2003; Dondo et al., 2006; Dondo et al., 2007; Rossi et al., 2007; Bergagna et al., 2009; Montagnaro et al., 2010; Magnino et al., 2011), an homogeneous and stable health monitoring is not still available. This fact should be considered in relation to the significant increase of wild ungulates recorded during the last decades (Pedrotti et al., 2001; Carnevali et al., 2009) and to the expanding anthropization, land-use change and *global warming* that contribute to an increase in interactions, mainly in built-up areas, between wild, domestic animals and humans favouring also health risks. Moreover the extent of both hunting plans and scheduled management programmes to limit overabundance of wild ungulates' populations has shown a general increase with a consequent rise of game meat consumption (Ramanzin et al., 2010). Considering that each hunting season around 155˙500 and 9500 of respectively wild boars and red deer are harvested with related about 5287.0 and 570.0 weight tons of meat available and that a 44-Kg wild boar (completely eviscerated) feeds about 147 people and a 88-Kg deer gives like 265 portions (Winkelmayer, 2010), public health issue takes on still more importance. In this regard, although until now health guaranteed for consumers' protection are aspects still deficient in our Country (Citterio et al., 2011), a strict monitoring of hunted animals should be planned and carried out including risks related to the microbiological

contaminations of carcasses, favoured by hunters' manipulations, whose the control is still at the very beginning (Citterio et al., 2011; Avagnina et al., 2012).

As regards parasitic food-borne zoonoses, there are specific statutory provisions concerning *Trichinella* sp. (Romano et al, 2011) laying down health control measures for all the hunted wild boars (Reg. E.C. 2075/2005). These measures are not provided for *Toxoplasma gondii* although it has the highest human incidence among the parasitic zoonoses (EFSA, 2007) with a total of 1˙259 confirmed human cases in 2009 (Lahuerta et al., 2009) and is one of those diseases which should be monitored according to their epidemiological situation (Directive 2003/99/EC, acknowledged in Italy by D.L. 191, 4th April 2006). Despite that, no Italian representative data are available for *T. gondii* either in humans or animals, either about its circulation in meat or in food products.

Beside domestic animals (Dubey, 1992; Masala et al., 2003), many wild species (Conrad et al, 2005; Bártová et al, 2006; Sobrino et al, 2007; Jokelainen et al, 2011) can act as intermediate hosts helping to maintain both domestic and sylvatic parasite lifecycles (De Craeye et al., 2011) and favouring the widespread distribution of the infection. Indeed *T. gondii* viable cysts were detected in the musculature of roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*), mule deer (*Odocoileus hemionus*), pronghorn (*Antilocapra americana*), and moose (*Alces alces*) (Vikøren et al, 2004) rising a public health issue related to *T. gondii* zoonotic potential. Considering the substantial amount of game meat available from harvest plans every year and that approximately half of the game produced in Europe may be seropositive for *T. gondii* (EFSA, 2007; Kijlstra and Jongert, 2009), the consume of raw or undercooked game meat or fresh sausages (typical products) (Dubey and Beattie, 1988; Ross et al, 2001; Gauss et al, 2006; Kijlstra and Jongert, 2008; Park et al, 2011) or the manipulation, evisceration and handling of carcasses (McDonald et al., 1990; Dubey 1994; Kapperud et al., 1996) should be evaluated as risks for human infection. Moreover *T. gondii* zoonotic potential must be considered in relation to its three major multilocus genotypes, types I, II, and III, described by several genetic studies of isolates from Europe and the United States (Robert-Gangneux and Dardé, 2012). Indeed different *T. gondii* strains have a contrasting level of pathogenicity in humans (Robert-Gangneux and Dardé, 2012), type I strains are uniformly lethal while type II and III strains are significantly less virulent (Howe et al., 1996). In Europe *T. gondii* type II is highly predominant while type III is more present in South Europe, asymptomatic or benign disease in immunocompetent individuals are associated with these two types including a lower rate of retinochoroiditis in immunocompetent patients and in those with congenital toxoplasmosis than in South America (Robert-Gangneux and Dardé, 2012). However new *T. gondii* atypical genotypes were associated with higher rate and severity of retinochoroiditis in immunocompetent patients, in those with congenital toxoplasmosis and in disseminated,

potentially lethal, cases (Robert-Gangneux and Dardé, 2012). Considering that these new genotypes appear to take origin to wildlife, since recombination of genetic material seems to occur more frequently in the sylvatic cycle (Carme et al., 2002; Ajzenberg et al.,2004; Grigg and Sundar, 2009; De Craeye et al., 2010), *Toxoplasma gondii* monitoring in wildlife takes on still more importance.

Among *T. gondii* intermediate hosts, many wild species were investigated and showed different receptivity and susceptibility to the pathogen. Despite the well documented toxoplasmosis zoo-economic losses in goat and sheep (Duncanson et al., 2001; Masala et al., 2003; Pereira-Bueno et al., 2004), little is known about the epidemiology of *T. gondii* infection in wild ungulates' populations particularly regarding the possible impact of the parasite on populations' dynamics considering the documented role of pathogens on wildlife welfare and demographic shrinkage (Hudson and Dobson, 1995; Ebert et al., 2000; Citterio et al., 2006). Although vertical transmission recorded in white tailed deer (Dubey et al., 2008) and natural transplacental toxoplasmosis documented in a stillborn *R. tarandus* foetus (Elmore et al., 2012) point out the hypothesis that also red deer could be affected, no assessments are reported about *T. gondii* associated-reproductive pathologies in this species and just few studies have investigated the effect of parasitism on fecundity of ungulates in natural conditions (Pioz et al., 2008). Nonetheless a well-documented and serious impact of *T. gondii* was recorded on hares (*Leporidae, genus Lepus*) and Eurasian red squirrels (*Sciurus vulgaris*) populations, species exceptionally susceptible to primary infection. In this regard *T. gondii* assumed a conservation value since a few cases of fatal toxoplasmosis were described in these species (Jokelainen et al., 2011; Jokelainen and Nylund, 2012; Jokelainen, 2012).

The described different *T. gondii* potentialities in wildlife give evidence to the need of monitoring the infection within free-ranging populations although several diagnostic issues rise in this regard. Indeed no gold standard test for the screening of the large diversity of *Toxoplasma* host species (Robert-Gangneux and Dardé, 2012) is still available, for domestic animals too. In addition, considering that usually diagnostic tests applied to wildlife were sharpened from domestics, the difficulty to compare results from screenings of free-ranging population samples takes on more significance because different methods, both serological and molecular, each with their own sensitivity and specificity, are used (De Craeye et al., 2010). As this regards the need is to identify and standardize accurate and sensitive testing methods defining a certified diagnostic trial with serological and molecular examinations that allow to detect infected animals though all the production chain in order to evaluate the actual health risk for consumers and to provide management tools to prevent the circulation of the disease (Hill et al., 2006).

Aims of the study

- Sero-epidemiological investigation of *Toxoplasma gondii* in monitored populations of red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), alpine chamois (*Rupicapra r. rupicapra*) and wild boar (*Sus scrofa*), with an evaluation of the agreement between the ELISA test performed with sera and tissue fluids in the first three host species.
  - Epidemiological investigation to define factors that can influence the spread of the infection within studied populations.
- Molecular investigation: detection of *T. gondii* DNA applying three PCR protocols in series;
  - genotyping of *T. gondii* to evaluate the different pathogenic significance and the potential zoonosic risk linked to the consumption of meat.
- Microbiological examinations (Total Aerobic Counts, *Enterobacteriaceae* and *Salmonella* spp.);
  - collection of superficial samples of carcass standardized sections;
  - deep tissue samples collected from the external section of the haunch muscle.

Toxoplasma gondii investigation

Material and methods

The survey has been carried out in two different Alpine study areas, similar in level of direct/indirect anthropization but different in extent and in host species population sizes.

Study area and sampling collection

Ossola valley (Lepontine Alps)

Sampling collection was strictly connected to hunting seasons (in accordance with the Italian Law 157 of 11/02/1992) and was carried out in the Alpine hunting Territory (hereafter referred as A.T.) VCO2 (http://www.vco2.it/) in the province of Verbania which has an extent of 72.740 ha and includes the Formazza, Antigorio, Cravariola, Isorno and Vigezzo valleys. In the study area wild ungulates populations are present and standard censused annually: alpine chamois is the most represented with a density of 5 subjects/kmq, red deer with a population size of 2 subjects/kmq, roe deer of 2.5 subjects/kmq and wild boars have a sporadic presence in the territory. Even if hunting activity on this latter host species is not well-established in the study area, wild boar was included in the sampling activities because of its well-documented seroprevalence and role in the *T. gondii* lifecycle (Gauss et al., 2005).

Hunters were previously be involved in the project since a test-tube was given to each of them in order to collect blood when the animal was bleeding, directly from the major blood vessels. Once hunters carried culled animals to the A.Ts to declare their shooting and for a post mortem inspection of carcasses, the test-tubes had been delivered and placed in the centrifuge and the serum was stored in eppendorf tubes at -20°C. Moreover hunters voluntarily let us collect heart, if it's present, and brains that were immediately stored at -20°C.

From the whole sample of 245 sera (Table 1), morpho-biometric data and georeferencing of the place animals were shot was recorded for all the subjects while age of 236 wild ungulates (98 alpine chamois, 75 roe deer, 62 red deer and 1 wild boar) were registered since, in a few situations, hunters preferred keeping the hunted animals intact without cutting their cheeks for the exact teeth determination. Table 2 shows the number of animals which have been completely sampled through the collection of blood sample and the removal of brain tissues.

Species	Blood	Heart	Brain
Red deer	65	10	46
Alpine chamois	98	14	50
Roe deer	78	14	49
Wild boar	4	1	1
TOT	**245**	**39**	**146**

Table 1: Overall samples collected in Ossola valley during the three hunting seasons for each species

Species	N°
Red deer	5
Roe deer	15
Alpine chamois	10
TOT	**30**

Table 2: Number of animals which have been completely sampled (blood and brain tissue) during the three hunting seasons

Valfurva

The study area lies in Italian Central Alps in the Lombardia sector of the Stelvio National Park (http://www.stelviopark.it/), which borders the Brenta-Adamello Natural Park and the Engadin National Park. The sampling area, which is located in Valfurva (Province of Sondrio; Long 10,41; Lat 46,48) spans 1400 ha, encompasses an elevation range from 1300 to 2400 m a.s.l. and is characterized by spruce and larch forests, calcareous alpine pastures and hay meadows around human settlements in the lower part of the slopes. In the study area inside the Park, red deer shows very high population density: in Valfurva and in the neighbouring hunting districts, where standardized annual census are performed, in spring 2012 around 1300 subjects were counted and densities inside the Park ranges between 12 deer/Kmq according to summer distribution to more than 40 deer/Kmq in wintering areas (official report of Stelvio National park 02/13/2012). According to the strong impact that red deer exert on forest regeneration and biodiversity, the National Park had defined a management plan to reduce red deer density and to monitor its effects on biodiversity conservation.

The study area was divided into two different sub-areas considering both the geographical and morphological distinction of natural boundaries. The two sub-areas (1 and 2) are respectively 772 ha and 707 ha wide. In sub-area 2 human settlements and cultural landscapes (i.e. hay meadows around villages and hamlets) are much more distributed and represent the 32% of the total surface

(226 ha), while in sub-area 1 they represent a smaller fraction of the whole surface (7%). According to the ongoing study based on marked and radio-tagged deer, the two sub-area are clearly permeable to deer movements, but a traditional and stable use of space within each area has been outlined, especially for hinds and calves. Because of the considerable recorded densities, red deer habitually inhabit areas close to human settlements, including gardens and hay meadows, in proximity to private houses, farms and touristic activities (restaurants, mountain dews, hotels), especially during winter and spring nights.

Here, in addition to the epidemiological investigation on the overall population, we investigated:

- if *T. gondii* infection, acquired by hinds during the breeding season, in September or in October early in pregnancy (Mustoni et al., 2002):
  - could cause early abortion and drive dams to lose the breeding season;
  - may have an effect on the physiological foetus development;
- the sera prevalence of *T. gondii* IgG antibodies in domestic cats to understand the level of horizontal contamination in the study area.

<u>Sampling collection</u>

Red deer

Sampling collection was carried out during the culling management plan, scheduled by the staff of the park and carried out by formed hunters, in January and February 2012 and during two consecutive weeks between the end of November and the beginning of December 2012. For each culled deer, the shooting area (site, altitude, exposure, etc.) was registered and a post mortem inspection of carcasses was performed to verify their healthiness. Morpho-biometric measures were recorded relatively to body weight, total length, jaw length, hind foot length, withers length, neck circumference and Kidney Fat Index (KFI). Age class was estimated by examination of tooth wear in lower jaw. In females, lactation and pregnancy were also registered together with foetus morpho-biometric measures (body weight and length) to evaluate the gestational age in concordance with Adam et al. (1988).

Blood samples were collected directly from major blood vessels during animal bleeding, then test-tubes have been placed in the centrifuge and the serum had been immediately stored in eppendorf at -20°C. Hearts, foetus and milk samples were collected during evisceration while brain tissues were gathered later at the guaranteed slaughterhouse using the same technique of removal the obex of domestic animals. Samples (Table 3) had been immediately stored at -20°C.

	Sera	Heart	Brain	Foetus	Milk
January-February 2012	24	22	24	-	-
November-December 2012	89	46	89	33	43
TOT	113	68	113	33	43

Table 3: overall samples collected in Valfurva during the two culling management plans

Cats

A total of 23 sera were sampled from domestic cats which live in the previous described two origin areas. The subjects were 15 males and 8 females respectively 2 aged < 1-year old, 3 between 1 and 1.5- year-old while 18 were adults. We chose these sampled cats because they are used to spend all/the major part of their day outside in gardens, forests, grasslands and pastures in overlapping with the red deer population. Sampling took place going house-to-house and asking the permission of owners to let to draw blood from their cats.

Serological investigation

Toxoplasma gondii antibodies were detected in sera and cardiac tissue fluids through a commercial enzyme linked immunoabsorbent assay (ELISA test ID Screen® *Toxoplasmosis Indirect* MULTI-SPECIES) validated for ruminants and cats, already used for wild ungulates (Magnino et al., 2011). Test was performed according to manufacturer's instruction with different dilution for sera (1:10) and tissue fluids (1:2). Tissue fluids were collected from each heart by thawing at room temperature, then fluid collection was carefully cleaned of debris by filtration. For each sample the resulted values were calculated applying the formula supplied in the kit: (%S/P = (ODsample-ODnc / ODpc- DOnc) x100) and interpreted according to the guidelines (Table 4).

RESULT	STATUS
S/P ≤ 40%	NEGATIVE
40% < S/P< 50%	DOUBTFUL
S/P ≥ 50%	POSITIVE

Table 4: description of ELISA test results

Test agreement

Sera and tissue fluids of 78 red deer (10 from Ossola Valley and 68 from Valfurva), 14 roe deer (Ossola valley) and 14 alpine chamois (Ossola valley) were analyzed to test the level of agreement between the ELISA performed using the two matrices. As previous surveys showed 91.3% of clinical sensitivity and 98.7% of clinical specificity in ELISA from sera (Chong et al., 2011) in contrast with the 76% of sensitivity recorded using the test with tissue fluids (Hill et al., 2006), for each species the frequency data into the 4 cells (both tests positive; test 1 positive and test 2 negative; test 1 negative and test 2 positive; both tests negative) of a 2x2 table were recorded considering serum as the comparative test and tissue fluids as the alternative one. The concordance between them was evaluated by calculating the Kappa (K) value and the results were interpreted according to the guidelines (Sergeant, 2012; Table 5). The software EpiTools epidemiological calculators was used for this analyses (Sergeant, 2012).

Kappa Value Evaluation

> 0.8 - 1	Excellent agreement
>0.6 – 0.8	Substantial agreement
>0.4 – 0.6	Moderate agreement
>0.2 – 0.4	Fair agreement
>0 – 0.2	Slight agreement
0	Poor agreement
-1 - <0	Disagreement

Table 5: Kappa values associated to different levels of agreements between two tests

Statistical analysis

Ossola valley

T. gondii sera prevalence was calculated for each host species. Then the dichotomous variable infected and non-infected has been modelled with binomial distribution to investigate the epidemiology of the infection. The explanatory variables were sex (categorical), age class (categorical), year (categorical), hunting month (categorical) and habitat anthropization level (categorical). This latter factor was available by the georeferencing data of the place where animals were shot. In particular, considering the densities of human settlements and the anthropic presence during the year, culling localities were divided in high (1), moderate (2) and low (3) level of anthropization. Then pairwise comparisons (Sidak post-hoc test) were used to compare each

level of factors and interactions. The analyses were undertaken using SPSS Statistic 17.0® software; values were significant when $p<0.05$.

Valfurva

Three Generalized Linear Models were set up:

1. binomial GLM considering the dichotomous variable infected and non-infected red deer in the overall population has been used to investigate the epidemiology of the infection: the explanatory variables were origin area (categorical), KFI, age class (categorical) and sex (categorical);
2. the pregnancy status (dichotomous) was modelled with a binomial GLM in order to define the effect of hind serological titers, hind age class (categorical), hind KFI, hind origin area (categorical);
3. hinds' gestational ages were modelled with a normal GLM, including progressive days of foetus development, hind serological titers, hind serological titers: hind serological titers, hind KFI, hind lactation status (categorical), hind origin area (categorical), hind age class (categorical), hind lactation: hind KFI, hind serological titers: hind origin area, hind serological titers: hind age class as explanatory variables.

The interaction "hind serological titers: hind serological titers" was included because from a previous graphic check a non linear (parabolic) correlation among gestational ages and serological titers emerged.

Model were initially fitted till their second order interaction; then it was simplified by backward selection using AIC and AICC reference values to eliminate factors and variables that did not contribute significantly to explanatory power of the model; Sidak post-hoc test was used to compare each level of factors and interactions included. For each model the random distribution of residuals and their normality test were performed. The analyses were undertaken in SPSS Statistic 17.0® software; values were significant when $p<0.05$.

Serological titers resulted from different ELISA plates were made comparable through a standardization applying the following formula ((serological titer – cut-off value)/cut-off value) per each titer. Then a graphic check was performed testing the correspondence between each standardized values and the ones resulted from the above mentioned formula supplied by the ELISA manufacture's instruction.

Molecular investigation

Detection of T. gondii by PCR

DNA was extracted from brain tissues, foetuses, homogenized totally since they were small (about the size of a mouse) and bones were not ossified (Dubey et al., 2008), and from milk samples using QIAamp DNA Mini Kit (Qiagen, Italy) according to the manufacturers' recommendations.

All the samples were assayed by a polymerase chain reaction using the primers described by Homan et al. (2000), TOX4 (5'-CGCTGCAGGGAGGAAGACGAAAGTTG-3') and TOX5 (5'-CGCTGCAGACACAGTGCATCTGGATT-3'), that target a 529 bp non-coding region. The PCR reaction was performed in a 50 µl reaction mixture containing 1 X buffer, 0.5 µM of each primer, 0.1 mM dNTP, 2 mM $MgCl_2$, 0.1 U Taq (Promega, Italy) and 10µl di DNA.

A second PCR protocol (Hurtado et al., 2001) was used to confirm any positive/negative or doubtful results taking advantage of its increase in sensitivity and specificity. Indeed a previous survey about the molecular detection of *T. gondii* in coypus (*Myocastor coypus*) described false negative results using the protocol in concordance with Homan et al. (2000) and then detected by Hurtado et al. (2001) as reported by Zanzani et al. (2014, submitted). The Hurtado et al. (2001) method is structured with two successive amplifications performed in a single tube using primers that target the 18S-5.8S rRNA ITS1. The technique employs two pairs of primers, the external ones are NN1 (5'-CCTTTGAATCCCAAGCAAAACATGAG-3') and NN2 (5'-GCGAGCCAAGACATCCATTGCTGA-3'), and the internal primers are Tg-NP1 (5'-GTGATAGTATCGAAAGGTAT-3') and Tg-NP2 (5'-ACTCTCTCTCAAATGTTCCT- 3').

The 25 µl reaction mixtures contained 2 µl DNA, 1 X buffer, 10mM Tris–HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.2mM of each deoxynucleotide, 0.1 µM of each external primer, 0.4 µM of each internal primer and 0.5 U Taq polymerase (Promega, Italy).

A third PCR-RFLP protocol according to Magnino et al. (1998) was performed to confirm the positivity and to exclude eventual cross-reactions with *Neospora caninum* and *Sarcocystis spp.* since the employed primers identify all the three protozoa. Primers API-F (5'-AAGTATAAGCTTTTATACGGC-3') and API-R (5'-CACTGCCACGGTAGTCCAATAC-3') target the 18S small-subunit ribosomal gene of *T. gondii.* Amplification was performed in a final volume of 20 µl with a final concentration of: 1 X buffer (Promega, Italy), 1.5mM $MgCl_2$, 0.2mM of each deoxynucleotide, 0.7 µM of each primer and 1 U of *Taq* polymerase. Then the amplified fragment were digested with *Bse*DI at 55°C for at least 3h.

Genotyping analysis

T. gondii genotype was determined by the characterisation of the SAG2 gene (I, II or III) using a PCR-RFLP (Howe et al., 1997). In particular, two nested PCRs were performed separately amplifying the 5' and 3'- ends of this gene: the 5' end of the locus was amplified with the primers SAG2.F4 (5'-GCTACCTCGAACAGGAACAC-3') and SAG2.R4 (5'-GCATCAACAGTCTTCGTTGC-3') while the second amplification was performed with the internal primers SAG2.F (5'-GAAATGTTTCAGGTTGCTGC-3') and SAG2.R2 (5'-GCAAGAGCGAAC TTGAACAC-3'). The amplified fragments were digested with *Sau*3AI, and the restriction fragments were analyzed by 2% agarose gel electrophoresis.

The 3' end of the locus was similarly analyzed with the primers SAG2.F3 (5'-TCTGTTCTCCGAAGTGACTCC-3') and SAG2.R3 (5'-TCAAAGCGTGCATTATCGC-3') for the initial amplifications and the internal primers SAG2.F2 (5'-ATTCTCATGCCTCCGCTTC-3') and SAG2.R (5'-AACGTTTCACGAAGGCACAC-3') for the second round of amplification. The resulting amplification products were digested with *Hha*I, and analyzed by 2% agarose gel electrophoresis.

DNA from *T. gondii* parasite strains served as positive controls were kindly provided by the Pavia division of the Lombardy and Emilia-Romagna Experimental Institute of Zooprophylaxis "Bruno Ubertini".

Results and Discussion

Serology

Comparison between sera and tissue fluids

A *T. gondii* prevalence of 29% (23/78; 95% CI: 39-19) and 19% (15/78; 95% CI: 28-10) was recorded respectively in red deer sera and tissue fluids samples. In roe deer a prevalence of 35.7% (5/14; 95% CI: 61-11) in sera and of 42.8% (6/14; 95% CI: 69-17) in tissue fluids were registered. No positive results were recorded in chamois.

Red deer		**Tissue fluids**		**TOT**
		+	-	
Sera	+	**10**	**13**	**23**
	-	**5**	**50**	**55**
Total		**15**	**63**	**78**

Table 6: Results yields from the comparison of the two tests in red deer

Roe deer		**Tissue fluids**		**TOT**
		+	-	
Sera	+	**4**	**1**	**5**
	-	**2**	**7**	**9**
Total		**6**	**8**	**14**

Table 7: Results yields from the comparison of the two tests in roe deer

Alpine chamois		**Tissue fluids**		**TOT**
		+	-	
Sera	+	**-**	**1**	**1**
	-	**-**	**13**	**13**
Total		**-**	**14**	**14**

Table 8: Results yields from the comparison of the two tests in chamois

Red deer analysis showed a Kappa value of 0.38 (95% CI: 0.15–0.61) pointing out a “fair agreement” between the two tests while roe deer samples resulted in a K=0.55 (95% CI: 0.11-0.99) with “moderate agreement” between the two matrices. As regards alpine chamois, we were

not able to calculate the K value since none of sera samples resulted positive thus the comparison was not allowed.

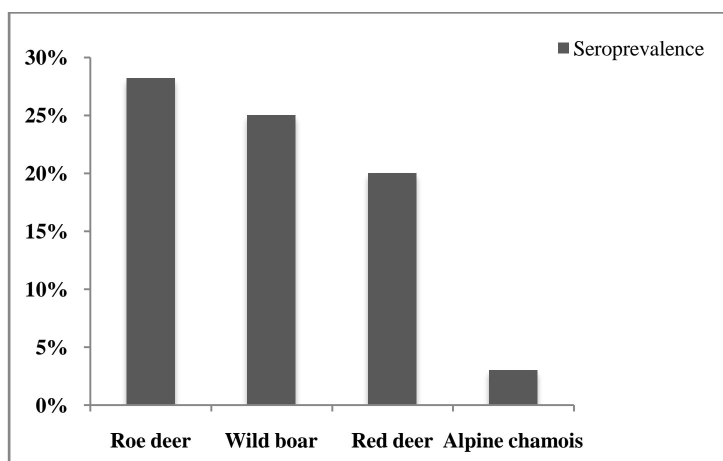
The agreement recorded between the two matrices for both red deer and roe deer excludes the consideration of tissue fluids as an alternative to serum for *T. gondii* epidemiological screening using this test. Although each sample had been carefully purified from coarse elements ensuring the greatest possible clarity, the presence of major protein component in the tissue fluids cannot be excluded interfering with the binding between the antigen and the antibodies favouring a discordance between the two tests. Therefore these results confirm the discrepancy in sensibility and specificity between sera and tissue fluids previously reported for *T. gondii* (Hill et al., 2006; Chong et al., 2011). As regards roe deer analyses, it must be underlined that the limited sample size (n=14) could not allow to draw firm conclusions about the real level of agreement between the two matrices.

Further analysis should be carried out considering that an ‘‘almost perfect’’ concordance between serum and diaphragm meat juice analyses using IFAT in wild boar was recorded (Ranucci et al., 2013). Therefore hearth tissue could not be the best choice, at least in the red deer, or this ELISA could be less reliable than IFAT for this purpose.

Ossola valley (Lepontine Alps)

Results

The overall seraprevalence were 28.2% (22/78; 95% CI: 38-18) in roe deer, 25% (1/4; 95% CI: 67-17) in wild boar, 20% (13/65; 95% CI: 30-10) in red deer and 3% (3/98; 95% CI: 6-0) in chamois (Graph 1).



Graph 1: representation of the *T. gondii* seroprevalence of the four species examined in Ossola valley

Roe deer

Seroprevalence was analyzed in relation to gender, 24% (6/25; 95% CI: 41-7) in females and 30% (16/53; 95% CI: 42-18) in males emerged, concerning age classes 16.6% (3/18; 95% CI: 34-1) in yearlings, 29.6% (16/54; 95% CI: 42-17) in adults arisen while no positivity were recorded in calf (0/3). Regarding the level of anthropization of the origin area: 21% (5/24; 95% CI: 37-5) in the high, 43% (6/14; 95% CI: 69-17) in the moderate and 27.5% (11/40; 95% CI: 41-14) in the low one were registered.

No significant effects of genders, age class, levels of anthropization, year and hunting month (GLM $p>0.05$) were recorded on the probability to get infected (GLM $p> 0.05$).

Red deer

Seroprevalence was analyzed in relation to gender, 11.5% (3/26; 95% CI: 24-1) in females and 25.6% (10/39; 95% CI: 39-12) in males emerged, concerning age classes, 21.4% (3/14; 95% CI: 43-0) in calf, 28.5% (10/35; 95% CI: 43-14) in animals between > 2 year old arisen, while no positivity were recorded in yearling (0/13). Regarding the level of anthropization in the origin area: 18% (8/44; 95% CI: 29-7) in the high, 8% (1/12; 95% CI: 23-7) in the moderate and 33% (3/9; 95% CI: 64-2) in the low one were registered.

No significant effects of gender, age class, levels of anthopization, year and hunting month (GLM $p>0.05$) were recorded on the probability to get infected (GLM $p> 0.05$).

Considering the other two analyzed species, *T. gondii* seropositivity was recorded in one adult female wild boar and in three alpine chamois, respectively in one 1-year-old male and in two adult subjects, one male and one female.

Discussion

The seropositivities recorded in all the four host species proves the circulation of *T. gondii* in the study area.

As regards roe deer, although the comparison of results obtained by different serological tests could be questionable, the emerged prevalence is linear to the ones observed previously in different European countries (Vikøren et al., 2004; Gamarra et al., 2008; Gaffuri et al., 2006; Panadero et al., 2010; De Craeye et al., 2010). In spite of the limited sample size of calves (n=3), the negativities recorded in this category could lead to the hypothesis of horizontal transmission as the main route of infection even if the no significant effects of sex and age class on seroprevalence does not permit to exclude that vertical transmission could play a role in the spread of the infection in the study area as pointed out in Spanish roe deer (Gamarra et al., 2008). Moreover this

theory could be supported by the fact that the different level of anthropization of roe deer origin area appear to do not influence the infection.

Concerning red deer population, the described seroprevalence is linear to the one emerged from Kapperud (1978), Hejlíček et al. (1997), Vikøren et al. (2004), Gauss et al. (2006), Aubert et al. (2010) and Magnino et al. (2011), the only reference we could find about *T. gondii*-serology in red deer from Italian Alps. The no significant effects of sex, age class and level of anthropization of the red deer origin area together with the recorded seroprevalence in adults (28.5%) and calves (21.4%) and, in addition, the negative results in yearling suggest that vertical transmission could be the main route of infection in this host species.

Concerning alpine chamois (n=98), the observed seroprevalence (3%) was similar to the one (5%) arisen from the analysis of 236 subjects from Orobie Alps (Gaffuri et al. , 2006). Moreover the seronegativities in 43 samples from Western Alps (Gennero et al., 1993), lead to a scarce *T. gondii* circulation in this species. In this regards the different habitat-use of alpine chamois, that live in most remote areas far from human settlements and built-up areas, can support these results.

On the other hand, considering that in *Rupicapra pyrenaica* a systemic toxoplasmosis and a gram-negative sepsis that lead to death was recorded (Marco et al., 2009), it cannot be excluded a priori that the low seroprevalence detected in the Alps could reflect the susceptibility of this species to *T. gondii* infection and could represent the small part of subjects able to overcome the acute form. In this regard the well-documented impact of *T. gondii* on hares and squirrels (Jokelainen et al., 2011; Jokelainen and Nylund, 2012; Jokelainen, 2012) could confirm the hypothesis.

As regards wild boar, the limited sample size (n=4) does not let any evaluation about the presence and the epidemiology of the protozoan in this species.

Valfurva

Results

The overall seroprevalence was 39.5% (32/81; 95% CI: 50-29). In relation to gender, a seroprevalence of 44% (22/50; 95% CI: 58-30) emerged in females and a 32% (10/31; 95% CI: 48-16) in males. Concerning age class: 51% (20/39; 95% CI: 67-35) and 52% (11/21; 95% CI: 73-31) of seroprevalence were recorded respectively in >2 year-old subjects and 1 year-old red deer while in calves, one female was seropositive. In relation to origin area, a seroprevalence of 17% (2/12; 95% CI: 38-4) was recorded in origin area 1 and of 43% (30/69; 95% CI: 55-31) in origin area 2.

Concerning the three set up models:

1) binomial GLM: infected/non infected

	Wald Chi-Square	df	Sig.
(Intercept)	8.794	1	0.003
origin area	3.547	1	0.060
age class	8.98	2	0.011

Table 9: tests of model effects

	B	Wald Chi-Square	df	Sig.
(Intercept)	0.198	0.352	1	0.553
origin area 1	-1.623	3.547	1	0.060
origin area 2	0a	.	.	.
calves	-3.067	8.082	1	0.004
1 year-old	0.262	0.207	1	0.649
> 2 year-old	0a	.	.	.
(Scale)	1b			

Table 10: minimal linear model of factors that influence the probability to get infected

A significant effect of age class was recorded: the probability to contract infection is significant lesser in calves than in 1-year-old (GLM, pairwise comparisons: p=0.000) and >2- year-old (GLM, pairwise comparisons: p=0.000) deer (Table 10). No significant difference was recorded between the two latter age classes (GLM, pairwise comparisons p>0.05; Table 10). No significant effect of the origin area was recorded on the spread of the infection within population (Table 10).

As regards adult females (n=34), the results of set up models are reported below:

2) binomial GLM: pregnancy/no pregnancy

	Wald Chi-Square	df	Sig.
(Intercept)	7.705	1	0.006
hind serological titers	3.387	1	0.066

Table 11: tests of model effects

	B	Wald Chi-Square	df	Sig.
(Intercept)	-1.915	7.705	1	0.006
hind serological titers	-2.000	3.387	1	0.066
(Scale)	1a			

Table 12: minimal linear model of factors that influence the probability to be pregnant

No statistically significant effect of serological titers on hinds' probability to become pregnant was recorded (GLM p>0.005; Table 12).

3) the gestational age of hinds (n=27) was estimated to vary from 25 to 68 days:

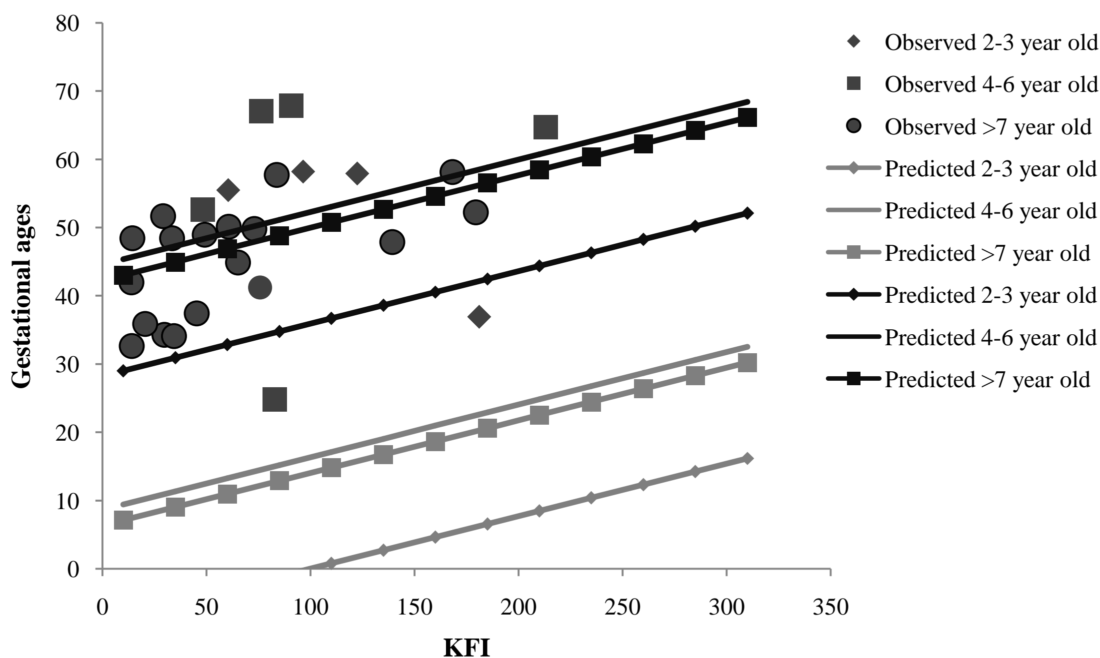
	Wald Chi-Square	df	Sig.
(Intercept)	117.49	1	0.000
origin area	17.192	1	0.000
age class	1.743	2	0.418
serological titers	26.642	1	0.000
progressive days of foetus development	44.479	1	0.000
KFI	24.922	1	0.000
serological titers: origin area	26.415	1	0.000
serological titers: age class	12.398	2	0.002

Table 13: tests of model effects

	B	Wald Chi-Square	df	Sig.
(Intercept)	31.371	354.569	1	0.000
origin area 1	-13.082	17.192	1	0.000
origin area 2	0a	.	.	.
2-3 year-old	-3.047	1.39	1	0.238
4-6 year-old	0.552	0.047	1	0.829
>7 year-old	0a	.	.	.
serological titers	1.178	0.681	1	0.409
progressive days of foetus development	1.245	44.479	1	0.000
KFI	0.077	24.922	1	0.000
serological titers: origin area 1	-28.022	26.415	1	0.000
serological titers: origina area 2	0a	.	.	.
serological titers:2-3 year-old	-13.49	10.112	1	0.001
serological titers:4-6 year-old	2.151	1.129	1	0.288
serological titers:>7 year-old	0a	.	.	.
(Scale)	11.384b			

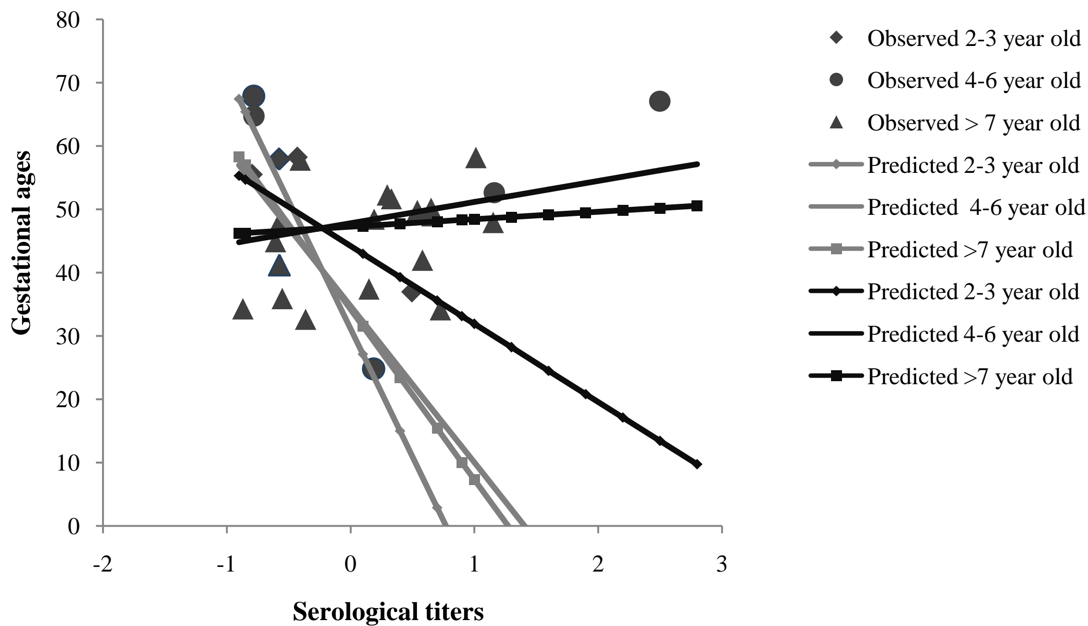
Table 14: minimal linear model of factors affecting the gestational ages of hinds

A significant positive effect of hinds' KFI on foetus development was recorded (GLM, $p<0.01$) (Table 14; Graph 2).



Graph 2: Gestational ages in relation to KFI. Light grey line: origin area 1; black line: origin area 2

The significant effect of origin area highlighted that in area 1 foetuses are significantly less developed than those from area 2. In addition, the model showed that an increasing of serological titers make foetuses from area 1 significantly smaller than those of area 2 ($p<0.001$) (Table 14; Graph 3). Concerning age class, a negative effect of serological titers on foetus development of 2-3 year-old dams ($p<0.01$) was recorded (Table 14; Graph 3).



Graph 3: Gestational ages in relation to “serological titers:origin area” and “serological titers:age class”. Light grey line: origin area 1; black line: origin area 2

Discussion

Serological investigation gives evidence to the circulation of *Toxoplasma gondii* in the study area and the equal infection of 1-year-old and >2 year-old red deer leads to the hypothesis of a high level of environmental contamination. Moreover a negative effect of serological titers on foetus development of 2-3 year-old females and of hinds from origin area 1 emphasizes a possible impact of *T. gondii* on the population dynamics.

The seroprevalence recorded is linear to the one emerged from Magnino et al. (2011) in Italian Alps and previous surveys from Spain (Gauss et al. 2006), France (Aubert et al., 2010), Czech (Hejlíček et al., 1997) and Norway (Kapperud, 1978; Vikøren et al., 2004).

In this research a high and equal level of *T. gondii*-infection in 1-year-old and adult red deer was recorded unlike previous surveys in both red deer and black-tailed deer (*Odocoileus hemionus columbianus*) that assessed a seroprevalence decreasing from yearlings to adults (Vikøren et al., 2004). This difference could be ascribed to an elevated level of environmental contamination, as

assessed also by the fact that animals in a short time (more or less 1 year) got infected, favouring horizontal transmission in our study area. Considering that lynx (*Lynx lynx*) has a still sporadic presence in the Italian Central Alps, transmission should be linked to the direct contact with soil, grass and drinking water contaminated with sporulated oocysts shed by feral and domestic cats, as pointed out in other different contexts (Vikøren et al., 2004; Gauss et al., 2006; EFSA, 2007; Gamarra et al., 2008; Jokelainen et al., 2010), with a high infecting power for their long-term survival (Kijlstra and Jongert, 2009). Apart just one female, calves did not contract *T. gondii* infection yet and this fact leads to the hypothesis of an almost total lack of vertical transmission in the studied population.

Concerning the origin areas, the absence of any differences in rate of infection could confirm a high spread of *T. gondii* within the overall population.

As regards factors influencing the probability of pregnancy, *T. gondii* infection seems not to have any impact on hinds to lose the breeding season and be apparently barren. Although the ELISA detection of IgG antibodies may not always permit an actual assessment of the moment of contagion and that previous *T. gondii* -infection gives an immunity that protects future pregnancies, at least in sheep (Hide et al., 2009), we hypothesized a hind early infection in the study area, considering that in domestic animals specific IgG antibodies reach a peak by day 35 (Conde et al., 2001) and that the production of specific IgG antibodies usually begins 4 weeks after the infection (Luptakova et al., 2012). In this regards, the non-significant effect of *T. gondii* could demonstrate that the pathogen does not cause early abortion or embryonic death and reabsorption in this species.

Nevertheless a *T. gondii* negative impact emerged on the foetus development of 2-3 year-old hinds, demonstrating that the pathogen could anyway affect their pregnancy. Young hinds could have contracted the infection likely at the end of summer and the pathogen could have delayed the breeding season, or cause an early abort and then re-get pregnant with a significant smaller foetus development. This latter hypothesis could be supported by the fact that if early abortion did not cause any complications *in utero* or to the hinds, as long as they do not become pregnant, they show a shortening of time between each estrus cycles (García et al., 2002) keeping on trying to get pregnant. In this sense it must be considered that free-ranging populations are not under strict control and any abortion or reproduction diseases could be undetected by the staff of the park. Otherwise *T. gondii* infection seems not to affect foetus development of 4-6 year-old and >7 year-old hinds. Considering that red deer seem to acquire *T. gondii* infection only temporarily eliminating quickly the parasite in early adulthood (Williamson and Williams, 1980; Vikøren et al., 2004) and that the EFSA (2007) suggests to restrict the testing for *Toxoplasma* to only young

adult red deer, older females could be more resistant to the infection. On the other hand the two above age class hinds could have more probability than 2-3 year-old ones to have contracted the infection during life and this result could be attributed to a long-standing immunity that besides protect them to congenital infection and abortion (Hide et al., 2009).
The recorded *T. gondii* negative effect on foetus development of hinds from area 1 could point out a more recent level of contamination of this area than the one of area 2. In this regard further analysis are desirable to deepen this hypothesis and to try to relate it with results, the preliminary ones are shown below, of the spread of *T. gondii* infection in domestic felids. Moreover considering that cats are the source of the infection in this study area, the difference between the two origin areas in cats' densities, that may vary between the municipalities within an area, and the related oocysts spreading in the environment should be evaluated.

Cats

Cats showed an overall *T. gondii* seroprevalence of 52% (12/23; 95% CI: 72-32). A value of 60% (9/15; 95% CI: 85-35) in males and 37.5% (3/8; 95% CI: 71-4) in females were recorded. In relation to age class the both < 1-year-old subjects resulted negative, while a seroprevalence of 100% (3/3) and of 50% (9/18; 95% CI: 73-27) emerged in respectively yearling and adults. Concerning origin areas, 2 subjects over 3 from origin area 1 and 10 over 20 analyzed from origin area 2 resulted *T. gondii* positive.
These data lead to an environmental contamination confirming the high prevalence registered in red deer. Even if the still limited cat sample size, the results recorded in 1-year old subjects give evidence to a recent shedding of oocysts in the study area.
Considering the elevated seroprevalence respectively in 1-year-old cats and adults, and the negativity recorded in the <1-year-old ones, even if only two subjects, the consumption of infected food or the access to potential intermediary hosts appears to be the *T. gondii* infection route. Moreover sheep and goat grazing in the study area, considering their well known susceptibility with abortion and stillbirth (Millán et al., 2009), could contribute as maintenance community (Haydon et al., 2002) to *T. gondii* diffusion and then favouring the infection in cats.

Molecular investigation

Ossola valley (Lepontine Alps)

T. gondii DNA was detected in one alpine chamois showing a whole prevalence of 2% (1/50). In particular, the protozoan DNA was detected by both used PCR protocols, Homan et al. (2000) and Hurtado et al. (2001), respectively reported in Fig. 1 and 2. Moreover the PCR-RFLP (Magnino et al., 1998) confirmed the presence of the pathogen and excluded eventual cross-reactions with *Neospora caninum* and *Sarcocystis spp.*, closely related to *T. gondii* (Fig. 3). Concerning the reliability of results, 10 chamois samples were submitted to the (Hurtado et al., 2001) PCR protocol since no-specific bands resulted using the first protocol (Homan et al., 2000). The negative results were hence confirmed.

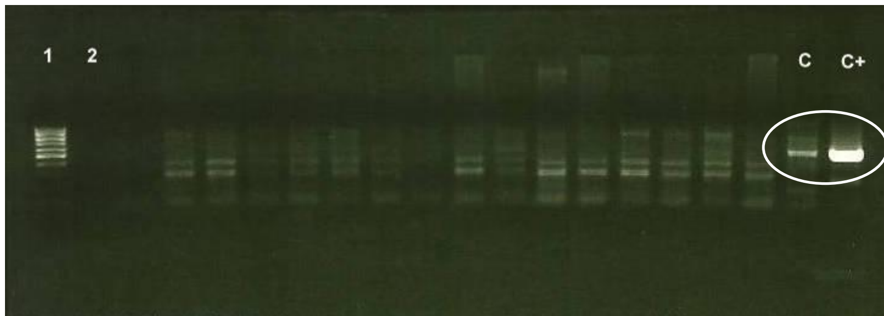


Figure 1: Agarose gel electrophoresis analysis of 529 bp non-coding region PCR amplification products. 1: molecular weight marker; 2: negative control; C: alpine chamois brain; C+: positive control RH strain

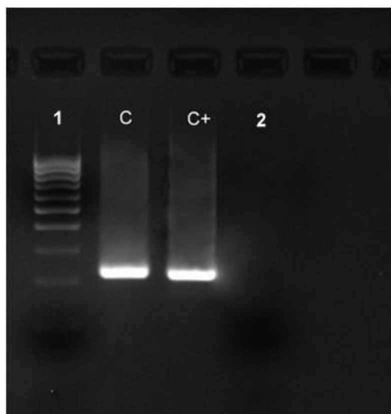


Figure 2: Agarose gel electrophoresis analysis of the 18S-5.8S rRNA internal transcribed spacer (ITS1) PCR amplification products. 1: molecular weight marker; C: alpine chamois brain; C+: positive control RH strain; 2: negative control

A) B)

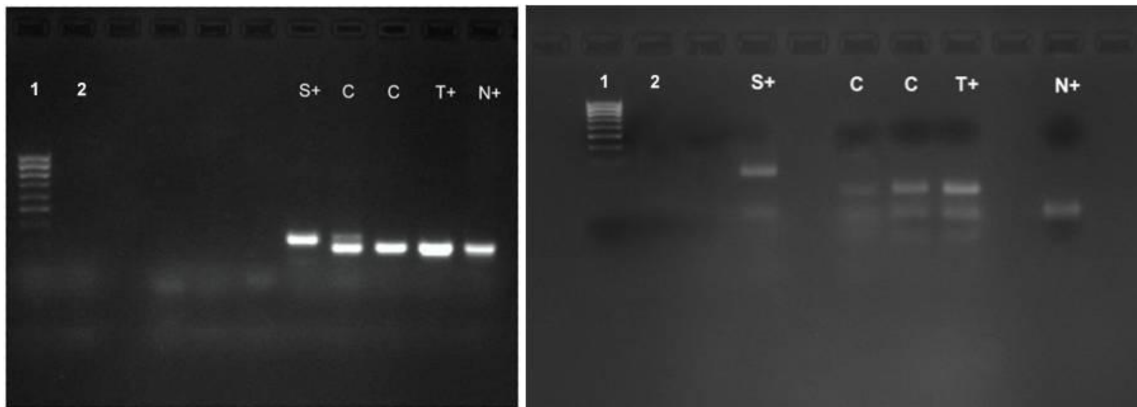


Figure 3: A) Amplicons obtained by PCR targeting the 18S rDNA; B) RFLP patterns obtained by *BseD* I digestion. 1: molecular weight marker; 2: negative control; S+: *Sarcocystis* spp. positive control; C: alpine chamois brain in double; T+: *T. gondii* positive control; N+: *N. caninum* positive

The subject was a six-year-old male hunted at an altitude of 1700 m a.m.s.l., in a good body condition and with normal behaviour; the post-mortem examination did not reveal any systemic macroscopic lesions. As far as we know, this is the first detection of *T. gondii* DNA from alpine chamois.

The result confirms this species as an intermediate host of *T. gondii* and demonstrates the protozoan presence in the Alpine ecosystem, even in remote areas.

The PCR positivity (p = 2%) agree with the recorded seroprevalence (p = 3%) confirming, on one hand, the hypothesis of a lower infection likely related to the remote habitat-use of alpine chamois. On the other hand the result could support the theory of a more susceptibility to the pathogen of this species.

As regards the other wild ungulates, molecular analysis was performed on 96 brain samples (46 red deer, 49 roe deer and 1 wild boar) and they all resulted negative. In 13 cases (5 in red deer and 8 in roe deer) no-specific bands resulted using the first PCR protocol (Homan et al., 2000), thus samples were submitted to the second one (Hurtado et al., 2001). Nonetheless the second PCR protocol cleaned the PCR amplified products and confirmed the negative results. These results agree with PCR negative previous surveys in wild ungulates in Italian Alps (Magnino et al., 2011) and the negativity in red deer and a low prevalence in roe deer in Belgium (De Craeye et al., 2010).

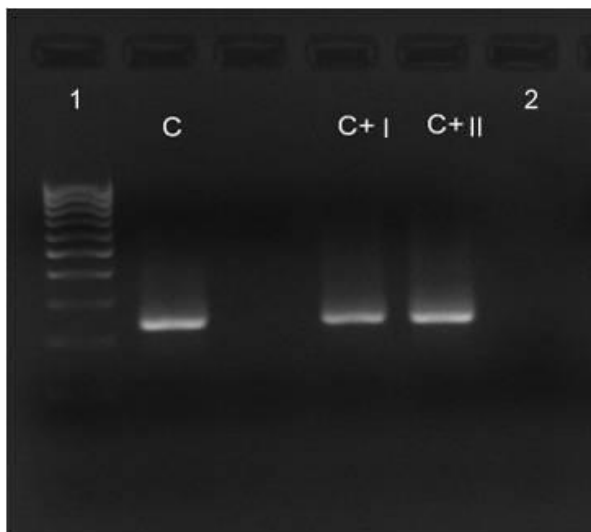
Concerning the above results it must be considered that developed PCR-based methods are less sensitive than bioassays due to the inhomogeneous, sporadic and random distribution of *T. gondii* tissue cysts (Hill and Dubey, 2002; EFSA, 2007; Robert-Gangneux and Dardé, 2012) and to the

small size of the tissue sample required for the tests may limit their sensitivity, usually 50 mg of sample for PCR assays, versus 50 to 500 g for bioassays (EFSA, 2007; Robert-Gangneux and Dardé, 2012). On the other hand the difficulties in the detection of pathogen DNA could be ascribed to some cases of pauciparasitism or a different susceptibility of the intermediate host. In this regards, even if roe deer is described to be particularly susceptible to *Toxoplasma* infection (EFSA, 2007), data emerged give evidence that for just 15 samples of sera and brain tissue available (see Tab.2), 12 resulted negative by both tests while in 3 roe deer ELISA positivity was not confirmed by PCR. Even if the limited sample size does not lead firm epidemiological assessment, the discrepancy between the above tests has been already described (Robert-Gangneux and Dardé, 2012). As regards red deer, the PCR negative results could be due to an apparent more resistance to the pathogen since this host seems to acquire the infection only temporarily and get rid of it in early adulthood (EFSA, 2007). In addition, ELISA and PCR confirmed the negativity for 5 samples whose both blood and brain tissue were available (see Tab.2).

Genotyping

The PCR–RFLP SAG2 revealed that the recorded *T. gondii* DNA was SAG2 type II. The first nested PCR (Figure 4) distinguished the type III strain from types I and II strains of the 5' end of the locus. Then the second nested PCR differentiated types I and III strains from type II strains of the 3' end of the locus (Figure 5).

A)



B)

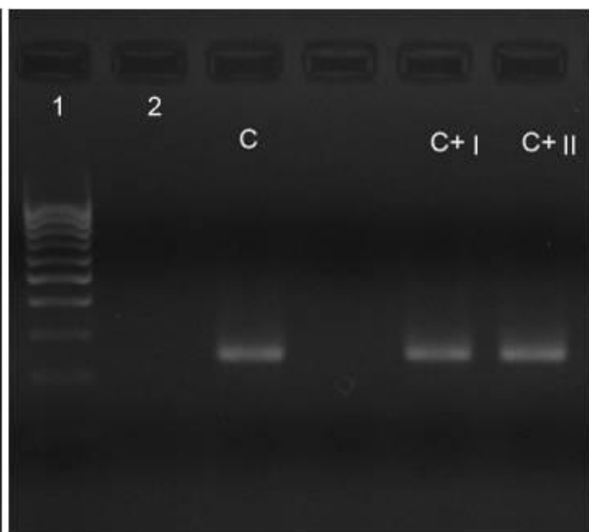
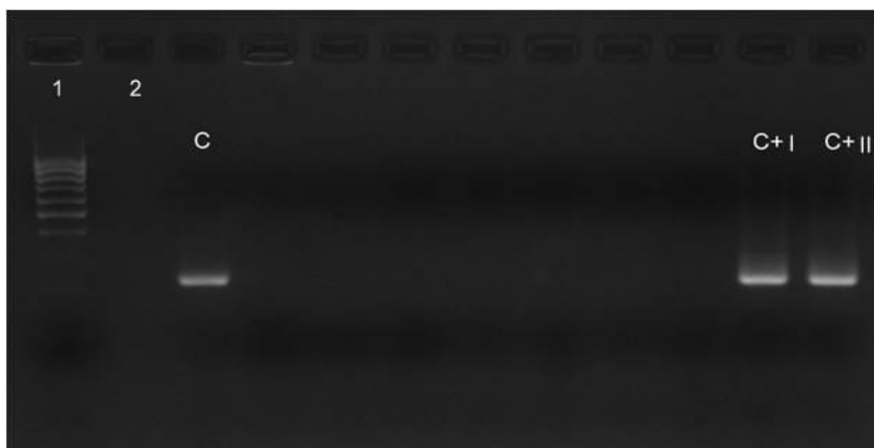


Figure 4: A) Agarose gel electrophoresis analysis of *SAG2* PCR amplification products; B) *Sau*3AI restriction analysis of the 5' amplification products. 1: molecular weight marker; 2: negative control; C: DNA from alpine chamois; C+I: positive control of type I;. C+II: positive control of type II

A)



B)

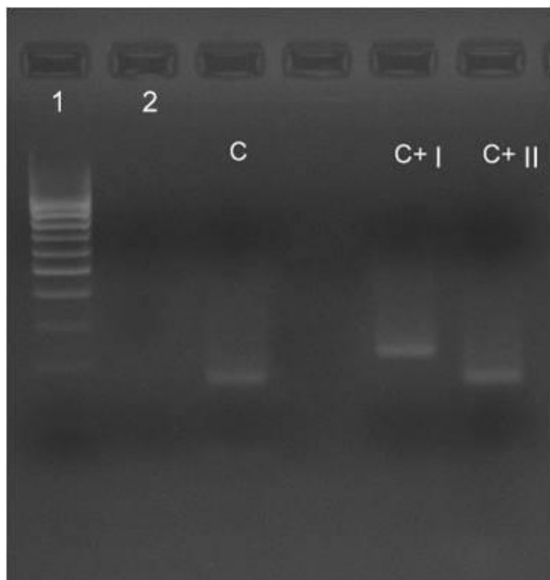


Figure 5: A) Agarose gel electrophoresis analysis of *SAG2* PCR amplification products; B) *Hha*I restriction analysis of the 3' amplification products. 1: molecular weight marker; 2: negative control; C: DNA from alpine chamois; C+I: positive control of type I;. C+II: positive control of type II

T. gondii type II is endemic in Europe (Jokelainen, 2012) and it was previously recorded in red fox, wild ungulates and mallards in France (Aubert et al., 2010); in European brown hares and red squirrels in Finland (Jokelainen et al., 2011; Jokelainen and Nylund, 2012); in carnivores in United Kingdom (Burrels et al., 2013). In addition, type II is the most prevalent in humans cases of toxoplasmosis in Europe (Gallego et al., 2006), particularly in immunocompromised patients (Fuentes et al., 2001), regardless of the underlying cause of immunosuppression, site of infection or outcome (Ajzenberg, 2010). Moreover the involvement of type II strain was recorded in congenital toxoplasmosis in France whatever the clinical presentation, in asymptomatic or subclinical cases and in severe neuro-ophthalmic involvement or fetal death *in utero* (Ajzenberg, 2010).

Considering that alpine chamois is the most hunted wild ungulate in the Italian Alps and that about 12.600 subjects are legally hunted every year with an estimated game bag over 180 weight tons (Ramanzin et al., 2010), this *T. gondii* PCR positivity must be considered in relation to its zoonotic potential. Even if an actual estimation of human risk is not available since the correspondent number of cysts in skeletal muscles are still unknown, this DNA detection should be take into account in case of consumption of raw or undercooked meat rather than in case of manipulation of carcasses (Tenter et al., 2000). Moreover the fact that *T. gondii* usually affects its hosts without producing any clinical signs (Marco et al., 2009) could make rise the zoonotic risk related to the apparent healthiness of chamois meat.

Valfurva

The PCR protocol in concordance with Homan et al. (2000) was performed on 113 red deer brain tissues, 33 foetus and 43 milk samples. In addition, the 33 hinds and their correspondent 33 foetuses and as much milk samples were submitted also to the second PCR protocol (Hurtado et al., 2001). All the analyzed samples resulted negative. In 10 cases over the 83 red deer analyzed just by Homan et al. (2000), protocol by Hurtado et al. (2001) confirmed the negativity.

As in two of these 10 samples the first PCR protocol (Homan et al., 2000) showed a doubtful result (Figure 7), in contrast with the no-specific bands emerged from other PCR products, they were submitted to the third PCR protocol (Magnino et al., 1998). Thus the two DNA extracted respectively from a 6-month-old male and a 1-year-old female, showed a positivity for *Sarcocystis* spp. and sequencing analysis identified *Sarcocystis hjorti from* the 6-month-old calf.

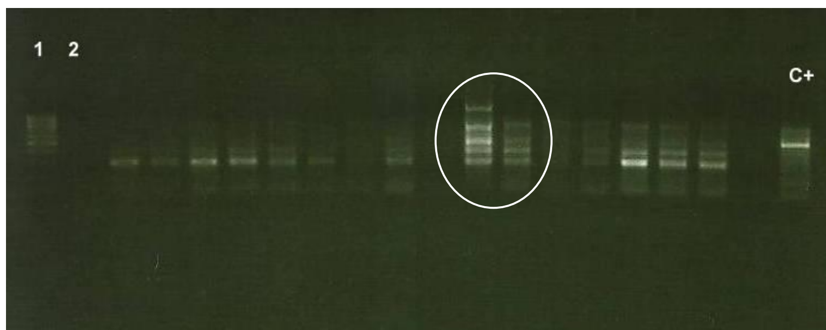


Figure 7: representation of the two doubtful results emerged performing the PCR protocol in concordance with Homan et al. (2000); C+: positive control

The emerged negative PCR results support what was mentioned above for Ossola valley in relation to the apparent red deer major resistance to the pathogen and to the difficulties of *T. gondii* PCR detection due to pauciparasitism infection or inhomogeneous, sporadic or random distribution of tissue cysts (Hill and Dubey, 2002; EFSA, 2007; Robert-Gangneux and Dardé, 2012). In particular, molecular analysis of hinds, their foetuses and milk samples points out that *T. gondii* seems not to have any vertical or other (milk) transmission confirming serological data. Moreover the fact that PCR analysis did not detect the pathogen DNA could support the hypothesis that red deer can control and get rid of the disease with a delay in mating or in pregnancy as the only detectable evidences.

The two false positivity emerged using our first PCR protocol (Homan et al., 2000) highlight that this molecular technique could have some diagnostic problems with red deer samples, demonstrating a low specificity that can be solved using PCR protocols in series.

Sarcocystis hyorti DNA was detected in the brain tissue of a 6-month-old male. The pathogen was recently described in Norwegian red deer and moose (*Alces alces*) (Dahlgren and Gjerde, 2010) with red fox (*Vulpes vulpes*), at our latitudes, and arctic fox (*Vulpes lagopus*) as definitive hosts (Dahlgren and Gjerde, 2010). Although is not a zoonotic pathogen, it must be considered that eosinophilic fasciitis caused by *S.hyorti* is described in two hunted red deer spoiled insomuch that carcasses have to be declared unfit for human consumption, according to meat inspection directives (Stephan et al., 2012).

Microbiological Examination

Material and methods

This research was been carried out from 2010 to 2012, during three hunting seasons, focusing on the investigation of the Total Aerobic Counts (TAC), *Enterobacteriaceae* and the presence of *Salmonella* spp. in carcasses of hunted wild boar. The sampled animals were from Alpine hunting Territory VCO 1 (Verbano Cusio) in the province of Verbania, mainly from Cannobina valley; subjects were carried to the A.Ts control centre and then stored in cold room (+4°C). Morpho-biometric measures, time of culling, time of evisceration, interval between hunting and cooling, number and placement of bullets shot, the presence of any wounds on the carcasses and the placement during the storage were recorded in a descriptive sheet for each wild boar. In addition, meteorological conditions (Max-min Temperature) of the hunting day were registered.

After not less than 12-hour-storage and skinning, the microbiological samples were collected according to the criteria used in slaughterhouses for domestic animal carcasses (decree-law 471/01/EC; Regulation 1441/2007). The procedure includes non-destructive superficial sampling using sterile pre-moistened sponges on a area of $100cm^2$ (10 cm on each side). Sponges have been used in 3 points standardizing the procedure and repeating it systematically for each carcass:

- **Haunch**: collection of samples from the haunch muscle;
- **Sirloin**: sampling area located between the two kidneys (sirloin);
- **Chest**: sampling area located on the chest inner wall.

For a deep tissues microbiological investigation, we gathered muscle samples with the objective of finding a possible heart microbial contamination. The sampling has been collected in the haunch, through a previously sterilized knife, on an area of $64cm^2$ (8cm on each side).

Through the superficial samplings we investigated:

- Total Aerobic Counts (TAC)
- *Enterobacteriaceae* (EB)
- *Salmonella* spp.

Using samplings of inner tissue we searched for:

- Total Aerobic Counts (TAC)
- *Enterobacteriaceae* (EB)

At the beginning of each hunting season a control of the microbiological contamination of the cold room (internal surface, floor and room walls) were performed investigating for TAC, *Enterobacteriaceae* and *Salmonella* spp..

The mean values of microbiological analysis were calculated for each carcass and the related level of contamination was evaluated in relation to information reported in the descriptive sheets.

To date in Europe there are no explicit guidelines about the microbiological limits for carcasses of wild game meat; consequently we used values defined by the Regulation (EC) No. 1441/2007 (Table 15) for superficial analysis while the referenced limits reported in the decree-law 471/01/EC (Table 16) for meat samples taken by the destructive method, considering carcasses of pigs as references.

	Limits	
	m	**M**
Total Aerobic Counts	4.0 log cfu/cm^2	5.0 log cfu/cm^2
Enterobacteriaceae	2.0 log cfu/cm^2	3.0 log cfu/cm^2
Salmonella spp.	Absent	

Table 15: Microbiological reference values laid down by the Regulation (EC) No. 1441/2007 for carcasses of pigs. TAC and *Enterobacteriaceae* in carcasses: satisfactory, if the mean log is ≤ m; acceptable, if the mean log is between m and M; unsatisfactory, if the mean log is > M

	satisfactory	**marginal**	**unsatisfactory**
Total Aerobic Counts	< 4.0 log	< 4.0 log – 5.0 log	> 5.0 log
Enterobacteriaceae	< 2.0 log	2.0 log – 3.0 log	> 3.0 log

Table 16: Microbiological reference values (ufc/cm^2) laid down by the decree-law 471/01/EC for pig carcasses samples taken by the destructive method

On the whole 38 superficial sponges and 31 deep tissue samples were gathered from wild boar carcasses. We could sample just from this species because hunters stored in the cold room their carcasses waiting for the result of the compulsory trichinoscopic examination; on the contrary they are not still used to storage carcasses of other wild ungulates regularity.

Samples have been carried to the Lombardy and Emilia-Romagna Experimental Institute of Zooprophylaxis "Bruno Ubertini" - Bergamo division - maintaining an unbroken cold chain

(+4°C). There they have been subjected to microbiological investigations within 24 hours after the gathering.

Results and Discussion

Data emerged from the first study year (2010) are not reported here since they were preliminary results that served to standardize the protocol and are difficultly compared to those registered from the other two hunting seasons.

Concerning TAC and *Enterobacteriaceae* of both superficial microbiological examinations and the analyses of the deep tissues, results are reported respectively in Table 17 and Table 18; *Salmonella* spp. have never been detected in meat during all the sampling years.

Wild boar carcasses	2011 (n=18)		2012 (N=9)	
	TAC	EB	TAC	EB
Log (cfu/cm^2) ≤ 5* (≤ 3**)	9 (50%)	7 (39%)	8 (88%)	8 (88%)
Log (cfu/cm^2) >5* (> 3**)	9 (50%)	11 (61%)	1 (12%)	1 (12%)
Median log cfu/cm^2	6.04	4.3	3.9	2.8

Table 17: Results from the superficial microbiological examinations of wild boar carcasses during all the sampling years. * limits for TAC and for **EB defined by Regulation (EC) No. 1441/2007

Wild boar carcasses	2011 (n=11)		2012 (N=6)	
	TAC	EB	TAC	EB
Log (cfu/cm^2) ≤ 5* (≤ 3**)	9 (81.8%)	7 (64%)	5 (83.3%)	1 (16%)
Log (cfu/cm^2) >5* (> 3**)	2 (18.2%)	4 (36%)	1 (16.7%)	5 (84%)
Median log cfu/cm^2	4	3	4.8	4.45

Table 18: Results from the microbiological examination of the inner section of the haunch muscle of wild boar carcasses during all the sampling years. * limits for TAC and for ** EB legislated by the decree-law 471/01/EC

The comparison between results of microbiological examinations and values defined by the Regulation (EC) No. 1441/2007 and by the decree-law 471/01/EC shows that the bacterial

contamination level of our samples is quite high. In particular, microbiological levels overtake the reference limits making meat "*unsatisfactory*" for human consumption in 12 subjects over 27 (44%) and 9 subjects over the 17 analyzed (53%) respectively in superficial and deep tissue analysis. These considerable bacterial contamination levels depend on a few incorrect hunters' manipulations, described in details below, that favoured the increase in the microbiological contamination of carcasses.

Hunting season 2011

In two carcasses we recorded an over-the-limit level of superficial microbiological proliferation since after the culling hunters eviscerated incompletely the animals, leaving in the chest heart and lungs, and waited for 8 hours before storing their carcasses during a day with a mean temperature of around 15°C. Then they stored their incomplete eviscerated carcasses using a hook fixed inside of the jaw instead of hunging them by the knuckles of ham: this incorrect placement applied to not-yet-skinned carcasses causes the retention of fluids, that were not allowed to be drained by gravity, in the muscle mass of the thigh (area of standard sampling) and together with the concomitant accumulation of blood and previous wrong manipulations favoured the rise of bacterial contamination. The same wrong placement were recorded in other 8 carcasses.

Concerning incorrect manipulation, other two subjects were stored in contact between them and in contact with the walls of the cold room: although this wrong placement is not a direct source of secondary contamination, such as the external environment, the contact with other surfaces prevents the carcass aeration and thus the loss of water, as well as to prevent a correct cooling process.

Data arisen point out that an incorrect shooting can favour the increase of TAC and EB bacterial proliferation: bullets wounds play a central role in the contamination of carcasses since "the entry bullet-hole" and "the exit bullet-hole" (if it is present) represent main access routes for the spread of bacteria, normally present on the skin or in the environment, into tissues: perfect *pabulum* for their proliferation. In 3 sampled wild boar we observed that animals were culled with more than one shot that damaged the carcasses near the sampling points (sirloin and haunch). In one of those the first bullet wounded the anterior right leg, then the second hit and spoiled the sirloin while in others two "the entry bullet-hole" was in the right haunch and the "the exit bullet-hole" in the correspondent in the left haunch contaminating meat. Moreover a rise of superficial microbiological counts were registered in two carcasses in which the abdomen was wounded leading to the intestinal contents discharge with meat contamination. In particular, in one of those an increase of bacterial proliferation in deep tissue was also registered: this animal was culled with a bullet that had hit in the haunch and then had exited from the abdomen spoiling the meat. After

the culling, hunter eviscerated the subject incompletely (leaving in the chest heart and lungs) and stored it in contact with the wall of the cold room, increasing further the level of bacterial contamination.

In other three wild boar carcasses the microbiological counts of deep tissue overcame the law limits because temperatures ranged from 15°C to 27°C during hunting days and a delay in cooling of around 7.30 in one and of 10 hours in others were recorded, in addition one of those was previously described since it was placement incorrectly too.

Hunting season 2012

The limits of TAC and EB of both superficial and deep tissue exceeded over the law values in one carcass hit by three shots before the culling: the first bullet wounded the posterior leg, others two had passed through the carcass in a longitudinal direction penetrating from the left side of the neck and remaining stuck in the muscles of the right thigh, damaging thus visceral organs. These facts, together with an interval of around 2.3 and 10 hours respectively of culling-evisceration and of culling-cooling, explain the high microbiological counts.

The same delay in culling-evisceration and in culling-cooling affected other four wild boar carcasses for which we recorded an overcoming of the law limits of deep tissue microbiological counts. Indeed hunters waited about 3 hours before evisceration and a mean value of 10 hours before storing during hunting days in which the mean temperature was around 20°C.

In addition it must be underlined that delayed, incorrect or incomplete bleeding contributed to the increase of microbiological counts recorded in the deep tissue analysis in both hunting seasons, mainly in warm days. Indeed, although the complete correctness of this practice could be difficult to be estimated, we observed that hunters had still some deficiencies in applying this good practice.

Conclusions

Traditionally wild game meat was used just for domestic consumption or for the preparation of local typical products but nowadays, favoured by the new European food hygiene regulations (Reg. EC. 852, 853, 854/2004), its consumption is increasing on a large scale. This fact takes on much importance considering the currently recorded great impact of human pressures on agriculture and natural resources with a progressive disrupting of ecosystems and climate (Foley et al., 2011; Hughes et al., 2013). In particular, agricultural systems are progressively degrading land, water, biodiversity and climate making rise the need to evolve to a food security and sustainability in which food production must grow substantially while, at the same time, agriculture's environmental footprint must shrink dramatically (Foley et al., 2011).

In this regard wild game meat represents a sustainable and high-qualitative product whose relaunch and an increasing of commercialization and consumption is desirable to contribute to reduce these anthropic pressures, mainly on intensive farming. However the promotion of these products require a strict health monitoring that allow to detect infected animals through all the food chain and to protect consumers from any risks, considering the well-documented role of wildlife in spreading zoonosis (Henaux et al., 2013; Michel et al., 2013). In particular, the definition of standardized and sensitive diagnostic testing methods is the starting point of this programme, although is not always easy because usually diagnostic tests applied to wildlife were sharpened from domestics compromising performances of tests and their reliability in most of the times.

In this research a *T. gondii* diagnostic trial defined on domestic animals was applied to wild ungulates and from that contrasting results emerged. Indeed *T. gondii*-type II DNA was detected in an alpine chamois for the first time, to our knowledge, by all the three performed PCRs. That points out a good sensitivity of protocols, genotyping test included, that allow the detection of the pathogen in this wild species. Considering the *T. gondii* zoonotic potential and the high popularity of chamois among hunted wild ungulates in the Italian Alps, this DNA detection should be taken into consideration in case of consumption of raw or undercooked meat rather than in case of manipulation of carcasses.

On the other hand a few diagnostic problems emerged in the molecular investigation of all the species, mainly for cervids. Indeed in a few cases the PCR protocol in concordance with Homan et al. (2000) showed a less specificity displaying doubtful PCR products and in two red deer from Valfurva *Sarcocystis* spp. DNA was recorded. In particular, after the sequencing analysis, from a six-month-old calf brain tissue was detected *Sarcocystis hjorti* DNA. On the contrary PCR according to Hurtado et al. (2001) was reliable confirming a doubtful or negative result, showing a

good specificity such as it emerged for the protocol described by Magnino et al. (1998), useful also to exclude any cross reactions with *N. caninum* and *Sarcocystis spp.*, closely related to *T. gondii*.

Apart the positivity recorded in chamois, the negativities registered in all other wild ungulates' samples point out that the monitoring of *T. gondii* chronic infection in asymptomatic subjects using PCR techniques appears to be difficult and in most cases gives negative results. On one hand PCR investigation can be useful for the detection of *T. gondii* DNA from an aborted or a stillbirth foetus or from viscera during an acute disease with systemic involvement. On the other hand direct PCR can have more difficulties in case of latent toxoplasmosis as previously mentioned (Hill and Dubey, 2002; EFSA, 2007; Robert-Gangneux and Dardé, 2012). In this regard mouse and cat bioassays are the most sensitive means of detecting cysts although these techniques are expensive, laborious and time-consuming thus poorly adapted to the screening of large numbers of samples (Robert-Gangneux and Dardé, 2012). From that it emerged that serology can have a central role in the screening of populations and in proving the circulation of the infection within populations. Although these tests can only demonstrate a contact between host and pathogen and the potential zoonotic role of animals cannot be assessed for certain, the detection of chronic infection with *T. gondii* relies primarily on serological assays (Robert-Gangneux and Dardé, 2012). In this regard it must be considered that attention should be given to the choice of the test and to the starting matrix: in fact we observed a not successfully level of agreement between the ELISA commercial kit performed from sera and tissue fluids, mainly for red deer, although it was validated for both matrices. Therefore, even if working with wildlife it could be easier sampling an heart than a serum sample, cardiac tissue fluids cannot be considered as an alternative of serum using this ELISA test. Nonetheless serology was very useful to investigate the presence of *T. gondii* infection within the analyzed populations, indeed data emerged from serosurvey prove the circulation of protozoan in both study areas with at least one positive subject recorded for every analyzed wild ungulate species. In particular, in the red deer population from Valfurva a high *Toxoplasma gondii* prevalence with horizontal transmission as the only route of infection was recorded and besides confirmed by the results emerged from the serosurvey in cats. In this regard the elevated population size in a relatively slight extent area could play a central role in this mechanism since not only host density is a well-known risk factor for many diseases but it was the main factor affecting seroprevalence of *T. gondii* infection in wild boars in Spain (Gamarra et al., 2008). On the contrary the lower seroprevalence recorded in red deer from Ossola valley with the supposed vertical or congenital transmission could lead to the hypothesis that the inferior red deer population size and the more extended area could be less favourable characteristics for the

spreading of the pathogen just through horizontal transmission. Further analysis should be desirable to deepen and understand the hypothesis including in the investigation other study areas with different host density and environmental conditions.

In addition, *T. gondii* analysis in Valfurva points out that the infection seems not to prevent red deer hinds to become pregnant although the recorded negative effect of the pathogen on foetus development of both 2-3 year-old hinds and females from origin area 1 points out that under determinate conditions *T. gondii* could give an impact on the population dynamics of this intermediate host. In this regard the negative results of PCR investigation on hinds, foetuses and milk seem to support the hypothesis of a possible resistance of this species to the pathogen with a delay in mating or in pregnancy as the only detectable evidences.

Microbiological analysis showed still a few considerable hunters' deficiencies in the application of hygienic practices for the management of culled animals. Although it is difficult that hunted carcasses have maintained a similar bacterial levels of those of slaughtered ones because of several logistic field problems, some increase of TAC and EB recorded in this research could have been easily avoided through a better manipulation from culling to cooling. Indeed sometimes the registered levels reached and overcame the law limits making meat unfit for human consumption or edible only after a long cooking period since such a microbial contamination could cause potential food poisonings. In particular, delayed, incorrect or incomplete bleeding, delay in evisceration or cooling and the related elevated temperature of the hunting day (Paulsen and Winkelmayer, 2004), a wrong placement of shot and a delayed death of animal or an incorrect placement in the cold room are the factors that affected the microbiological counts recorded in a few analyzed wild boar carcasses.

From this research it emerged that still a few aspects related to the protection of consumers and to the management of hunted carcasses should be improved to guarantee the healthiness of wild game meat products and to promote its spread on a large scale. On one hand a regular health monitoring of wild populations should be scheduled and, in this regard, a *T. gondii* serosurvey should be included in those areas in which their characteristics (land-use and environmental peculiarities) or human habits (people' culture, personal behaviour, socio-economic, health or alimentary habits (Rothova 2003; Demar et al., 2007)) could favour the contagious or the spread of the pathogen. In particular, human density and both domestic and wild animals' population sizes and the rising level of interactions between them and the well-established tradition of hunting and the related generational transmission of some practices should be taken into account in choosing the screened areas. On the other hand the need is to define and apply prevention programmes to increase the knowledge of both risking categories (hunters, slaughters, etc.) and

consumers about toxoplasmosis, considering the impossibility to detect routinely the pathogen in slaughtered carcasses and the lack of any legislation, and about all the other health risks related to wildlife. In this regard an increasing of involvement of hunting associations in continuing education courses can be good possibilities to prevent *T. gondii* infection, in both humans and animals, since it could be easily avoided through the application of the more common hygienic and sanitary practices (Kijlstra and Jongert, 2008; Robert-Gangneux and Dardé, 2012).

Although during years hunters' awareness about the importance of the health monitoring of wildlife have improved, a few deficiencies are still present mainly in relation to practices of manipulation of carcasses and their involvement in sampling activities. In this regard the definition of explanatory guidelines about the correct management of hunted carcasses, together with the regular involvement of hunters in continuing education courses, can be useful to keep on sensitizing and creating well-formed hunters who could be able to make the best use of the intrinsic valuable potentialities of wild game meat and at the same time commercializing healthy and qualitative products.

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