## Allegato 2a_ 5

# Brucellosis and infectious diseases did not drive the decreasing of Alpine ibex (*Capra ibex*) population in Gran Paradiso National Park (Italy)

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### Abstract

The presence and prevalence of n. 13 pathogens, potential causes of abortion or perinatal mortality in wild ungulates, were tested using serological analysis. From 1995 to 2012, 120 serum blood samples were collected by jugular venipuncture in free-ranging Alpine ibex (99 males and 21 females), captured by chemical immobilization in four different study areas of the Gran Paradiso National Park. All samples were negative for CAEV, BT, *Neospora caninum* and MAP *Mycobacterium avium* subsp. *paratuberculosis* (ELISA test). There was one antibody-positive result for *Leptospira* spp. for all tested serovars, IBR, *Mycoplasma agalactiae* and *Brucella abortus melitensis*. Seropositivities were found for MAP (AGID test, n= 2), *Brucella ovis* (n= 2), *Besnoitia* (n= 4). Forty three ibex (P=0,46) were positive for *Salmonella abortus ovis-abortus equi* with titers ranged from 1:20 to 1:160. Three serum samples were positive for *Toxoplasma gondii*, while 5 serum samples were positive for BVD (P=0,07). All serum samples tested by VN test were negative for BVD and BD. The present serological survey showed that none of the tested diseases drived the decreasing of the original Alpine ibex population, occurred in the last 20 years.

## Introduction

The Alpine ibex population of Gran Paradiso National Park (GPNP) is the only original present in the Alps, since this specie almost went extinct in the 18th century with less than 100 individuals left in the Gran Paradiso area (Apollonio *et al.*, 2003; Grodinsky & Stuwe 1987). The dynamic of the population of GPNP has been monitored since 1956 (Jacobson *et al*. 2004; Mignatti *et al*. 2012). This population reached its historical maximum density in 1993 with 4,991 animals and then, in the last years, dramatically decreased, arriving at a minimum census size of 2.321 in 2009 (Hardenberg *et al*., in prep.). This decline has been recently related with the fall of the kid survival, which has dropped from about 70% in 1980 to 26% today (Hardenberg *et al*., in prep.). Several hypotheses were tested to explain this decline: the effect of climate change (Pettorelli et al., 2004 ), changes in population age structure, with progressive aging of adults linked to the lack of snow (Hardenberg *et al*., in prep.), the possible presence of pathogens able to reduce the kid survival, as described in other wild ungulates (Pioz *et al*., 2007; Marco *et al*., 2007 and 2008). Many bacterial and viral infections can affect Alpine ibex (Marreros *et al.,* 2011), among others in Italy have been described seropositivity for *Leptospira* spp. and pestivirus (Gennero *et al*., 1993), *Mycobacterium avium pseudotuberculosis* (Ferroglio *et al*., 2000), brucellosis (Bassano and Ferroglio, 2002) and two clinic cases of brucellosis infection were found in the GPNP population (Ferroglio *et al*., 1998; Ferroglio *et al.,* 2001). Recently a sierological survey on Swiss Alpine ibex populations revealed the absence of abortive infection (Marreros *et al*., 2011), so our goal was to describe, by serological analyses, the health status of GPNP population, in order to exclude the presence of abortive infections or infectious diseases that can affect young survival.

## Materials and methods

*Study area*

Serum samples were collected in four different study areas of the Gran Paradiso National Park (PNGP North-western Italian Alps; 45° 25' N, 07° 34' W): Valsavarenche, Cogne, Rhemes and Orco valley, in the framework of a long term research on Alpine ibex started in 1999 and still ongoing (Bassano *et al*. 2003; Grignolio *et al*. 2004; von Hardenberg *et al.* 2007).

*Animals and samples*

From 1995 to 2012, 120 serum blood samples were collected by jugular venipuncture in free-ranging Alpine ibex (99 males and 21 females), captured by chemical immobilization, using a

mixture of Ketamine and Xylazine, reversed by Atipamezole, following the capture procedure described in Bassano *et al*. (2004).

*Laboratory analysis*

Serum samples were separated by centrifugation at 2,000 x G per 10 min and stored at -20°C until laboratory analysis, carried out at the Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta of Turin. The list of tested antigens and methods used was showed in table 1. Thirty-six serum samples were tested for pestivirus (n. 36 for BVD and n. 15 for BD) at the University of Milan. Thirty-six sera were also tested by VN test for the presence of BVDV antibodies (n. 36 samples) against BVDV strain NADL (ATCC- VR 534) on Madin-Darby Bovine Kidney (MDBK ATCC CCL-22) and BDV (n. 16 samples) strain Moredun on Brescia bovine embryo kidney (BS/BEK BS CL 94). Two-fold dilutions of sera, heat inactivated at 56 °C for 30 min, were tested by incubating serum dilutions with virus suspension containing 100 TCID50 for 1 h at 37 °C in 5% CO2. The plates were incubated for 4 days for BVD and for 7 days for BDV. The antibody titers were expressed as reciprocal of the highest dilution that completely inhibited virus infectivity. Samples with antibody titre >1:4 were defined as positives.

*Statistical analysis*

The animals were classified by class-age (young, adults and old), sex and capture site.

The EPI INFO ver.6 statistical package was used to compare prevalence data among sex, age and site of capture with a $\chi^2$ test. Differences in prevalence were considered as significant when $p<0.05$.

## Results

All samples were negative for CAEV, BT, *Neospora caninum* and MAP *Mycobacterium avium* subsp. *paratuberculosis* (ELISA test). There was one antibody-positive result for *Leptospira* spp. for all tested serovars, IBR, *Mycoplasma agalactiae* and *Brucella abortus melitensis*. Seropositivities were found for MAP (AGID test, n= 2), *Brucella ovis* (n= 2), *Besnoitia* (n= 4). Forty three ibex (P=0,46) were positive for *Salmonella abortus ovis-abortus equi* with titers ranged from 1:20 to 1:160. Three serum samples were positive for *Toxoplasma gondii*, while 5 serum samples were positive for BVD (P=0,07). All serum samples tested by VN test were negative for BVD and BD (Tab. 1). Differences in prevalence among classes (age, sex and capture site) were not statistical significative.

Table 1. Prevalence of infections and methods used to detect evidence of contact with pathogen in Alpine ibex (*Capra ibex ibex*) in Gran Paradiso National Park, between 1995 and 2012.

| AGENT | POSITIVE | PREVALENCE | 95% C.I. | METHOD |
|---|---|---|---|---|
| MAP (AGIDT) | 2/120 | 1,7 | 0,5 –5,9 | OIE manual of diagnostic tests and vaccines for terrestrial animals 6th edition 2008 |
| MAP (ELISAT) | 0 | 0 | 0 | POURQUIER IDEXX laboratories |
| Brucella abortus/melitensis | 1/120 | 0,8 | 0,2–4,6 | According to Alton et al., 1988 |
| Brucella ovis | 2/120 | 1,7 | 0,5 –5,9 | According to Alton et al., 1988 |
| Neospora caninum | 0 | 0 | 0 | IDVet neosporosis indirect multi-species |
| Toxoplasma gondii | 3/120 | 2,5 | 0,9–7,1 | IDVet toxoplasmosis indirect multi-species |
| Mycoplasma agalactiae | 1/120 | 0,8 | 0,2–4,6 | IDEXX screening |
| Salmonella abortus ovis/equi | 44/120 | 37 | 29–46 | OIE manual of diagnostic tests and vaccines for terrestrial animals 6th edition 2008 |
| Leptospira | 1/120 | 0,8 | 0,2 –4,6 | OIE manual of diagnostic tests and vaccines for terrestrial animals 6th edition 2008 |
| Besnoitia | 4/120 | 3 | 1,3 –8,3 | IDVet screen bresnoitiose indirect |
| BVD | 5/120 | 4,2 | 1,8–9,4 | LSIVet ruminant BVD/BDp80-serum/milk |
| IBR | 1/120 | 0,8 | 0,2–4,6 | CHEKIT Infectious Bovine Rhinotracheitis Antibody Test Kit (screening)- IDEXX |
| BT | 0 | 0 | 0 | Bluetongue antibody test kit-IZS Teramo CdR |
| CAEV | 0 | 0 | 0 | IDEXX Antibody test kit-Maedi Visna/CAEV |

## Discussion

Considering that the decline of the ibex population was related to drop in kid survival from 70% in 1980 to 26% today, one can speculate that are the agents could be involved in this decrease.

This serological survey was related to animals captured between 1995 and 2012 when the GPNP population suffered its greatest decrease. Based on the results of the last census (2013= 2710 Alpine ibex) we tested 4,4 % of the population.

Our study demonstrated the absence of abortive agents (CAEV, BT, *Neospora caninum*, *Leptospira* spp., *Brucella abortus*, and *Brucella melitensis)* in GPNP Alpine ibex population.

The absence of seropositivity to CAEV, recently reported in French Alpine ibex population (Erhouma *et al*., 2008), as well to BT, could be related to the low contact rate between Alpine ibex and domestic sheep in GPNP. *Neospora caninum, a* protozoan parasite of canids, like grey wolves and coyotes (*Canis latrans*), but not red fox (*Vulpes vulpes*), may also infects wild ruminants (Woods *et al.*, 1994; Dubey *et al.*, 1999; Ferroglio & Rossi, 2001). A previous serological survey on Alpine ibex, carried out in the late '90, showed the presence of this infection in GPNP, with low prevalences (Ferroglio *et al.*, 2001). The current absence of the infection indicates the reduction of the presence of infected dogs and flocks and the effectiveness of the prohibition of entry of dogs within the protected area.

The absence of positivity for Leptospirosis in our sample could be explained both by the absence of infection in domestic animals, both by the ibex space use: for most of the summer they select very high altitudes and low temperatures reduce the survival of leptospires. These results contrast with those obtained in Abruzzo National Park (Italy), where the presence of *Leptospira*, *interrogans*, *australis* and *icterohaemorragiae* serogroup, was recorded in Abruzzo chamois (*Rupicapra pyrenaica ornata*) population (Gentile *et al.*, 2000; Rossi, *pers. com.*). Even in these cases, no effect of the infection on survival and population dynamics were described.

Very important was the absence of positivity towards *Brucella abortus-melitensis* (1/120, P = 0.8). The only positive sample belonged to an old male ibex survived from the first and last case on infection recorded in 1998 with the isolation of *Brucella melitensis* (Biovar 3, Ferroglio *et al*., 1998). The authors described the infection in a 7-years-old male ibex (in Orco Valley, GPNP), with clinical sign of brucellosis and serologically positive. Further studies demonstrated the presence and the persistence of this infection in GPNP with a very low prevalence (Bassano and Ferroglio, 2002), however it has been shown that transmission of infection from Alpine ibex and goat and sheep was negligible (Ferroglio *et al*., 2007). Present data demonstrated the disappearance of infection in GPNP population without drastic sanitary control measures and this confirmed the observation generally made in Europe: wild species are not the reservoir of infection and when brucellosis were eradicated from livestock, it quickly disappeared in wildlife (Simon and Sarrazin, 1992; Leon Vizcaino, 1991; Remetzova, 1964).

Only 2 serum samples resulted positive for antibodies to *Brucella ovis.* This infection showed high prevalences in rams during 2003 and many flocks, that usually share mountain summer pasture with wild ungulates, officially brucellosis-free since 1996, actually resulted infected (Gennero, *pers.com*.). These results demonstrated the poor attitude of the infection to switch from domestic to wild ruminant in wild.

It is still unclear the abortigen role of *Toxoplasma gondii* in wild mammals (Hill *et al.*, 2005) and the low prevalence recorded in our study area suggested that this disease can not influence the survival of Alpine ibex kids. A similar seroprevalences was also recorded for another infection related to the presence of domestic carnivores: Besnoitiosis. This disease may infect a wide range of vertebrate, including reptile, birds and mammals, like horses, rodents, goat, antilopes, reindeer and caribou (Mehlhorn *et al.,* 2009), but its pathogen role for wildlife is still unclear.

*Salmonella abortus ovis* and *Salmonella abortus equi* had the highest prevalence in our study area, but the recorded titres (titres 1:20, 1:40, 1:80, 1:160) demonstrate the absence of an active infection. These positivity probably were explained by previous contact with the pathogen without an active infection, but, nevertheless, they demonstrated a wide spread of *Salmonella* in the wild. Other studies demonstrated that *Salmonella* sp. infection is an emerging disease in bovine, but could involved many wild animals (badgers, birds, wild ungulates and rats) and human and human salmonellosis is one of the most common and economically important zoonotic disease (Chomel *et al.*, 2007). To date, there is no evidence of effects of *Salmonella* sp. on survival and reproduction of wild ungulates.

The prevalence of MAP, *Mycobacterium avium* subsp. *paratuberculosis*, evaluated both with AGID and indirect ELISA, was very low, but the distribution of this infection varies by geographic area and the diagnostic technique used (Tolari *et al*., 1987; De Meneghi *et al*., 2000; Gennero *et al.*, 1993; Ferroglio *et al*., 2000). However the role played by wild ungulates in the epidemiology of MAP is still poorly understood, as well as the effects of this disease on the population dynamic. The susceptibility of farmed red deer to the bovine strain of *M. avium subsp. paratuberculosis* was confirmed to be high (O'Brien *et al*., 2006) and it seems that roe deer are able to maintain infection. Contamination of Alpine ibex by sympatric cattle cannot be overlooked because some studies showed the possibility of a rapid diffusion of the disease in different ruminant species, both wild and domestic (Ferroglio *et al*., 2000). Previous studies demonstrated the high susceptibility of *Caprinae* to bovine origined MAP and the risk of infection is increased by the high diffusion and resistance of the pathogen agent on the pastures (Ferroglio *et al*., 2000). The prevalence of IBR infection in wildlife is poorly documented in continental Europe. Thiry *et al.* (1988) showed very low prevalence in wild ruminants in France and Belgium and no positivity was recorded in Switzerland in Alpine chamois, Alpine ibex, fallow deer and red deer (Engels, 1986). As previously described in Italy (Gennero *et al.*, 1993), also in our study the prevalence is very low and these result suggested a cross reactivity with the Cervid Herpesvirus 1 (CvHV-1, formerly Herpesvirus of cervidae type 1) or Caprine Herpesvirus 1 (CpHV-1, formerly bovid herpesvirus 6, BHV-6). Despite widely reported in wild

ungulates in North americans National Parks, with prevalences of 55% in wild cervids (Aguirre *et al*., 1995), there are no evidences of effects of this disease on population dynamics.

The low prevalence of BVD infection reported in this study contrasted with the high rate recorded in Piedmont region (Italy) among domestic ruminants, because of an actually endemical diffusion in cattle (Second European Symposium on BVD control, 2004). These results are in accordance with previous studies conducted in Bavaria and Austria, where the sympatric presence of wild ruminats and cattle, with a high prevalence of infection, did not correspond to the transmission of the disease (Frolich *et al*., 1998). The occurrence of BVD specific antibodies and the virus isolation from wild ruminants could suggets that wild ungulates might be a hidden reservoir of virus, creating serious problems for the national eradication plans actually applied in many European countries (Lindberg *et al*., 1999). Other studies demonstrated the relationship between the massive spread of BVD in cattle and the occurrence in sympatric roe deer population in Germany, but the sequence analysis of BVD isolated in roe deer indicated distinct strain, probably typical of roe deer and circulating in wild species independently of domestic livestock (Frolich *et al*., 1998). The very low prevalence of pestivus recorded in this study contrast with other researches done on mountain ungulates (mainly BD) that shown a detrimental effect for reproduction and juveniles (*e.g*. Pyrenean chamois: Pioz *et al*., 2007; Marco *et al*., 2007 and 2008). But, even if most of these studies deepen the presence and prevalence of pestivirus, also describing new and specific viral strains, the role of these infections as regulation factors remains doubt, in the absence of complex and integrated studies on population dynamics.

## Conclusion

Despite the population dynamics of Alpine ibex in GPNP were strictly monitored, with two censuses per year since 1956, as well as the health status, the effects of pathogens on this dynamic were difficult to demonstrate. Our serological survey showed, however, that none of the tested diseases, potential cause of abortion in wild ungulates, drived the decreasing of the original Alpine ibex population.

Actually, this result was not surprising, given that the population trend of the sympatric Alpine chamois was different, with a progressive increase of the animals, confirming that these infections, also affecting Alpine chamois, were not present in the study area or were distributed with irrelevant prevalence. Another important result was about *Brucella melitensis* because the outbreak, described at the end of the 90s, remained confined in the starting site, did not affect other neighboring valleys and ended up without drastic interventions. These results suggested that natural populations, with high values of average individual heterozygosity, were able to

eliminate the infection in one single generation, by the intervention of environmental regulation factors, first of all, snow cover and strong winter (Jacobson *et al*., 2004). The low presence and prevalence of infections transmitted by domestic animals could also be related to the Park conservation policies, which tend to reduce the presence of domestic animals (dogs and domestic ruminants) in many districts of the protected area. This situation was amplified, over the past 20 years, by the progressive reduction of livestock activities in the Park and in the surrounding areas.

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