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# Tolerance to Tick-Borne Diseases in Sheep: Highlights of a Twenty-Year Experience in a Mediterranean Environment

Elisa Pieragostini, Elena Ciani,
Giuseppe Rubino and Ferruccio Petazzi
*University of Bari*
*Italy*

## 1. Introduction

The European landscape is characterised by a range of diverse farming systems. These relate not only to varied geographical environments and animal genetic resources, but also to different social and cultural contexts for farming and food production. This diversity is unique to Europe and, among the European countries, Italy is the home for a great variety of native breeds because of its complex orography and its long boot shape with very different climatic conditions from north to south. In the 1980's, two of us moved from northern Italy to Apulia and soon came to appreciate the differences between the biotic and abiotic features of northern environment and the Apulian one. One of the most impressive differences were the enzootic tick borne diseases (TBD) and the related responses of the animals. As a consequence, much of our professional life has been devoted to the challenges posed by the diseases and to the study of the genetic peculiarities of native breeds both *per se* and in terms of their tolerance to TBD.

This report is a review of the results obtained in a 20-year experience investigating the haematological features and tolerance to tick-borne diseases in Mediterranean native sheep breeds - mainly Apulian native breeds - compared to exotic breeds under various experimental conditions. In the wake of William Thomson (Lord Kelvin), a pioneer in thermodynamics and electricity, who said in 1891 that when you can measure what you are speaking about, and express it in numbers, you know something about it, but when you cannot measure it, your knowledge is of a meager and unsatisfactory kind, the central concept or research theme that guided all our research efforts stems from the notion that direct measurement of disease phenotypes and/or physiological features such as the hematological pattern provides a direct assay for measuring disease changes and the attitude of a genetic pool in facing disease. The work is concerned with the following main issues:

- Haematological pattern of Apulian native sheep breeds
- Breeds and tolerance to TBD in Apulia
- Response to experimental anaemia
- Response to *Anaplasma ovis* infection in experimentally infected sheep.

## 2. Haematological pattern of Apulian native sheep breeds

In Apulia, the region covering the heel of the boot-shaped Italian peninsula, the rather harsh conditions of the soil and climate and the selective pressure of endemic haemotropic parasites have yielded genetic pools that are generally rustic and tolerant to the diseases caused by haemotropic parasites. An evaluation of the local genetic resources to explore their potential for sustainable and profitable genetic development programs is based on the knowledge of the physio-pathological features of blood according to species, breed and animal.

### 2.1 The Apulian sheep native breeds

Altamurana and Leccese, the latter also known as Moscia Leccese, are two ancient dairy breeds native to Apulia whose origins are not fully known. It is thought that they developed from an Asian breed, particularly from a Zackel type stock. They are rough wooled, well suited to life in harsh and semiarid conditions and they make good use of marginal pastures. Both breeds are seriously endangered. Though not as endangered as the former two breeds, Gentile di Puglia sheep may be considered, according to Alderson (2009), at risk of extinction because of their numerical scarcity and population trends. Yet, the Gentile di Puglia is classified as one of the main fine-wooled ovine breeds. The origin of the breed can be traced back to ancient Roman times when the soft fleece of an Apulian sheep, the Tarentine breed, was used to make the *togas* of important Roman citizens. According to William Youatt (1867), the Tarentine breed "had gradually spread from the coast of Syria and the Black Sea, and had now reached the western extremity of Europe. Many of them mingled with and improved the native breeds of Spain, while others continued to exist as a distinct race; and, meeting with a climate and a herbage suited to them, retained their original character and value, and were the progenitors of the Merinos of the present day."

### 2.2 Adult haematological pattern

Table 1 has been compiled from the existing repertoire of haematological values obtained analysing the blood of Apulian sheep; it reports least-square means (LSM) and standard error (SE) of haematological data obtained by analysing blood samples collected in population surveys of Gentile di Puglia and Leccese (Pieragostini et al., 1994; Pieragostini, 2006). Samples for Altamurana sheep were obtained from 58 purebred ewes ranging from 2-6 years of age and bred on an experimental farm near Bari (Pieragostini et al., 1999). Comparison with the literature (Jain, 1993), where range and medians are available, is also shown. On the basis of the normal probability plot, our data appear to follow a normal distribution where the median equals the mean.

When compared to normal blood values for sheep in the literature (Greenwood, 1977; Jain, 1993), the blood of Apulian sheep appears to be characterized by fewer erythrocytes that are normal in size and have higher haemoglobin content. This phenomenon typically seems to reflect a Mediterranean/North-African ovine blood picture (Pieragostini et al., 1994). The decreased PCV values correspond to lower blood viscosity and thus greater availability of water, which seems to be of particular adaptive significance in habitats characterized by an arid climate like Apulia (Ariely et al. 1986). The fact that some blood factors are related to the suitability of the breeds under particular environmental conditions was suggested long ago (Cresswell & Hutchings, 1962).

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| Parameter | Altamurana | | Gentile di Puglia | | Leccese | | Jain (1993) | |
|---|---|---|---|---|---|---|---|---|
| | LSM±SE | N | LSM±SE | N | LSM±SE | N | range | median |
| RBC ($10^6$/μl) | 8.3±0.13 | 58 | 9.4±0.06 | 263 | 8.3±0.14 | 145 | 9 - 15 | 12 |
| Hb (g/dl) | 9.8±0.11 | 58 | 10.4±0.25 | 263 | 9.3±0.32 | 996 | 9 - 15 | 11 |
| PCV (g/dl) | 30.6±0.33 | 58 | 30.6±0.16 | 263 | 29.5±0.11 | 996 | 26 - 45 | 34 |
| MCV (fl) | 37.9±0.52 | 58 | 32.8±0.13 | 263 | 36.4±0.59 | 145 | 28 - 40 | 34 |
| MCH (pg) | 12.2±0.15 | 58 | 11.2±0.05 | 263 | 11.6±0.21 | 145 | 8 - 12 | 10 |
| MCHC (g/dl) | 32.2±0.16 | 58 | 34.1±0.44 | 263 | 32.4±0.36 | 996 | 31 - 34 | 32 |
| WBC ($10^3$/μl) | 7.4±0.20 | 58 | 8.4±0.16 | 178 | 7.8±0.19 | 145 | 4 - 12 | 8 |

Table 1. Least-square means (LSM) and standard error (SE) of haematological data from adult animals belonging to native Apulian sheep breeds. RBC, Red Blood Cells; Hb, Haemoglobin; PCV, Packed Cell Volume; MCV, Mean Corpuscular Volume; MCH, Mean Corpuscular Haemoglobin, MCHC, Mean Corpuscular Haemoglobin Content; WBC, White Blood Cells; N, Number of animals.

Comparison of the data in table 1 shows that the haematological patterns in the three breeds are broadly the same. Gentile sheep seem to exhibit slight differences from the other two breeds, particularly as to the erythrocyte count, the mean corpuscular volume (MCV) and the mean corpuscular haemoglobin (MCH); in fact they are apparently the most European among the three. The traditional breeding sites of Gentile and Leccese differ substantially; one is in the southern part and the other in the northern part of Apulia, which is 500 Km long extending from the 39° to the 42° parallel. The Altamurana breeding site is in the Murgia uplands, in the central portion of Apulia. Its location in a rather harsh environment, together with the common origin of the two breeds, may account for the fact that Altamurana is closer to Leccese than to Gentile (Tab.1). However, a non-negligible point is that the physiological pattern characterizing the Altamurana and the Leccese breeds differs considerably from that of the Gentile di Puglia, as they belong to the group of dairy breeds while the Gentile is a fine wool and meat-producing sheep.

## 2.3 Lamb haematological pattern

Although the paucity of data in the literature concerning the haematological picture of lambs is scarce, general and particular information is available on the developmental pattern of their haematological values. The development of haematological picture of Altamurana lambs was investigated to assess the normal blood parameters and check the first occurrence in the blood smears of endemic endoerythrocytic parasites (Pieragostini et al., 2000). Standard haematological values were calculated for 22 Altamurana lambs controlled from birth to 18 months of age The values recorded in the neonatal period were strongly affected by birth weight. As clearly shown in table 2, the haemoglobin concentration (Hb), packed cell volume (PCV) and white cell count (WBC) exhibited significant age-dependent variations, particularly Hb % and PCV decreased while WBC increased.
Over weeks 1-5, red cell indices mainly followed the same trends as the Hb and PCV. Over the first four months, the RBC values on average remained unchanged at approximately 9 million/μl but then decreased. Starting from the fifth month, overall mean values were practically the same as in adults.

| Age | Haematological parameters | | | | | | |
|---|---|---|---|---|---|---|---|
| | RBC ($10^6$/$\mu$l) | Hb (g/dl) | PCV (g/dl) | MCV (fl) | MCH (pg) | MCHC (g/dl) | WBC ($10^3$/$\mu$l) |
| | LSM±SE | LSM±SE | LSM±SE | LSM±SE | LSM±SE | LSM±SE | LSM±SE |
| 2 days | 9.6±0.9 | 13.0±1.0 | 42.0±4.1 | 43.7±3.0 | 13.5±0.9 | 31.1±1.34 | 4.5±1.0 |
| 7 days | 8.7±1.1 | 12.6±0.9 | 39.7±1.9 | 46.2±6.0 | 14.7±2.2 | 31.7±1.17 | 5.4±1.6 |
| 15 days | 9.0±0.9 | 12.5±0.7 | 39.5±2.0 | 44.1±2.8 | 14.0±0.5 | 31.7±1.5 | 5.6±2.8 |
| 21 days | 9.4±0.8 | 11.6±0.7 | 35.7±1.5 | 38.2±3.32 | 12.4±1.0 | 32.5±2.1 | 5.3±1.7 |
| 30 days | 9.2±1.2 | 11.2±0.5 | 35.4±1.9 | 38.7±4.9 | 12.4±1.5 | 31.7±1.4 | 6.1±2.5 |
| 45 days | 9.9±1.2 | 10.8±0.8 | 35.5±2.0 | 36.1±3.8 | 11.0±1.4 | 30.5±1.9 | 7.5±2.1 |
| 2 months | 9.8±0.8 | 10.7±0.4 | 34.9±2.1 | 35.6±3.0 | 10.9±0.9 | 30.6±1.3 | 7.8±1.7 |
| 3 months | 9.3±0.8 | 10.5±0.4 | 32.7±1.4 | 35.4±3.1 | 11.4±1.0 | 32.2±0.9 | 6.8±1.8 |
| 4 months | 9.1±1.2 | 10.3±0.6 | 32.6±1.9 | 36.2±3.1 | 11.4±1.0 | 31.5±0.8 | 8.1±2.0 |
| 7 months | 7.8±0.5 | 9.1±0.5 | 29.9±1.4 | 38.4±1.3 | 11.7±0.6 | 30.6±0.8 | 7.9±1.9 |
| 9 months | 7.9±0.7 | 9.4±0.8 | 30.1±1.3 | 38.1±2.6 | 11.9±0.9 | 31.2±1.5 | 7.3±1.4 |
| 12 months | 7.5±0.3 | 9.4±0.5 | 28.5±1.5 | 38.0±2.1 | 12.4±0.6 | 32.8±1.3 | 8.9±1.4 |
| 15 months | 7.6±0.4 | 9.3±0.4 | 28.4±1.5 | 37.5±2.5 | 12.2±0.7 | 32.7±1.3 | 8.8±1.4 |
| 18 months | 7.8±0.3 | 9.3±0.3 | 28.6±0.7 | 36.8±1.0 | 12.0±0.5 | 32.6±0.7 | 8.6±1.4 |

Table 2. Least-square means (LSM) and standard errors (SE) of haematological values recorded for 22 Altamurana lambs controlled from birth to 18 months of age. Modified from Pieragostini et al. (2000). RBC, Red Blood Cells; Hb, Haemoglobin; PCV, Packed Cell Volume; MCV, Mean Corpuscular Volume; MCH, Mean Corpuscular Haemoglobin, MCHC, Mean Corpuscular Haemoglobin Content; WBC, White Blood Cells.

Considering that reference data are mainly from breeds originally selected in northern European countries, when a comparison was made between 12 month-old Altamurana lambs and their northern counterparts, the erythrocytes of the Altamurana were fewer (7.5 *versus* 11.8 millions/$\mu$l) but bigger (38.0 ft *versus* 26.5 ft) and full of haemoglobin (12.4 pg *versus* 9.3 pg). This is the same phenomenon encountered in Mediterranean/North-African ovine blood picture as well as in the native Apulian adults.

The overall pattern is suggestive of erythrocyte physiological effectiveness, which was confirmed by the perfect physical development of the subjects examined in this study. In the blood smears obtained at seven months of age, namely in full spring when lambs start to graze pastures, endoerythrocytic enzootic parasites (*Theileria* spp. and *Anaplasma* spp.) were recorded and then became a constantly occurring phenomenon as will be documented in the following sections.

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## 3. Breeds and tolerance to TBD in Apulia

Tick-borne diseases are of global importance to human and animal health and welfare. They are also responsible each year for dramatic economic losses which comprise direct losses from death of animals, loss of productivity and indirect losses due to the costs of control measures. In 1979, the amount of losses were estimated to be globally USD 7 billion (McCosker, 1979), but several reports on the economic costs of specific tick-borne diseases indicated that the earlier report is an underestimate (Jongejan & Uilenberg, 2004). There is a wide portfolio of measures which could be used to control tick-borne diseases among which both husbandry practices and host-related factors such as age, innate tolerance and breed are of great importance. Breeds whose historical breeding site is situated under the latitude of 41° show the ability to thrive in areas where tick borne diseases (TBD) are common. This trait, which can be defined as tolerance to TBD, is associated with the ability to resist the development of anemia in the face of infection.

A review on host resistance to tick borne diseases is documented in cattle (Correia de Almeida Regitano & Prayaga, 2010). As for other species, the case of the tolerance to tick-borne diseases shown by the sheep and horse native to Apulia is emblematic (Pieragostini & Petazzi, 1999; Rubino et al., 2006). In southern Italy, and particularly in Apulia, pyroplasmosis represents a longstanding and heavy burden for every type of livestock farm (Ceci & Carelli, 1999). Previous work performed on Gentile di Puglia sheep found that blood smears for parasite detection revealed an overall positivity rate of 93% for tick borne parasites (TBP) (Pieragostini et al, 2006). This high TBP positivity rate associated to normal blood values highlighted the tolerance of the native sheep towards TBP infection and accounted for endemic TBD.

### 3.1 Tick borne diseases in Apulian native sheep: A low income disease

According to Townsend & Thirtle (2001), studies of the rates of return to research have usually been based on the implicit assumption that if there were no research, then there would be neither growth nor decline in output or productivity. In the case of livestock, particularly in those areas characterized by a sub-tropical disease ecology, the assumption is especially unreasonable. It ignores the losses that would have occurred in the absence of livestock health research, resulting in an underestimation of the rates of return. The financial impact of a range of clinical and subclinical diseases and mortalities on farms is difficult to assess because there are insufficient accurate survey data on their prevalence causes or production losses on a national basis. Thus demonstration of the economic advantage of animal health is one of the relevant issues in animal production. Pieragostini et al. (1996) carried out a four year study to check the economic and zoonotic importance of TBD on sheep farms.

To this purpose sheep belonging to breeds tolerant to TBD systematically underwent one prophylactic treatment with diminazene aceturate (Berenil, Hoechst, AG, Germany) in full spring before the mating season. Table 3 shows the results obtained. The comparison between the reproductive values in the treated sheep and in an untreated control group highlighted significant differences in fertility and fecundity, with the group of treated sheep that were more fertile and fecund. Pyroplasmosis, even though unapparent, represents an important cause of perturbation of animal welfare. The authors estimated relevant (≈30%) economic losses in non treated animals, thus defining pyroplasmosis as a "low income disease".

| Parameter | Altamurana | | Leccese | | Total | |
|---|---|---|---|---|---|---|
| | T (N =149) | NT (N = 259) | T (N = 49) | NT (N = 89) | T (N = 198) | NT (N = 348) |
| Fertility (%) | 93[a] | 65[b] | 86[a] | 62[b] | 91[A] | 64[B] |
| Prolificacy (%) | 139 | 131 | 144 | 142 | 138 | 134 |
| Fecundity (%) | 132[a] | 89[b] | 119[a] | 90[b] | 128[A] | 89[B] |

Table 3. Least-square means of the reproductive parameters in Altamurana and Leccese sheep and in the whole sample (Total), as a function of the prophylactic treatment against pyroplasmosis with diminazene aceturate (T = treated, NT = not treated). Modified from Pieragostini et al. (1996). Means within rows with different letters significantly differ: capital letters: P< 0.001; small letters: P<0.05.

### 3.2 Breed sheep and TBD

It is now generally acknowledged that importing exotic breeds can result in activities within the livestock sector that are uneconomic and/or have a negative impact on the environment. In many cases these activities are subsidized or otherwise provided for by development programs such as the case of Apulia which was documented in a study assessing attempts to introduce highly productive north European sheep breeds to Apulia (Pieragostini & Petazzi, 1999). The investigation analyzed data concerning the incidence and severity of pyroplasmosis in the five years spanning 1980-1984 on an experimental farm situated on the Murgia uplands in the province of Bari. The farm contained sheep belonging to gene pools of different geographical origin (Apulian, Italian island and north European breeds) or genotype classification (pure breeds or crossbreds) (Fig. 1).

The northern Finnish, Friesian and Romanov breeds were very susceptible to the disease; conversely, the native Apulian breeds showed very low rates of morbidity and mortality, followed in turn by breeds like Sardinian and Comisana, whose native areas have climatic and pedological characteristics similar to those of Apulia (Fig. 2). It is also worth noting that while the native and island breeds were regularly taken out to graze, the north European breeds were kept constantly under cover to reduce the likelihood of encountering ticks.



1788    707
1145    128

☒ Apulian native breeds

▥ Italian islander breeds

■ Northern pure breeds
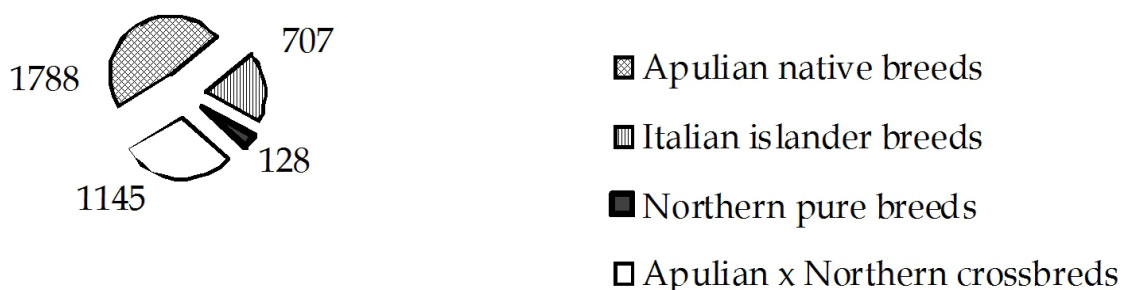
☐ Apulian x Northern crossbreds

Fig. 1. Size of the investigated samples, clustered as sheep ecotypes. Modified from Pieragostini & Petazzi (1999).
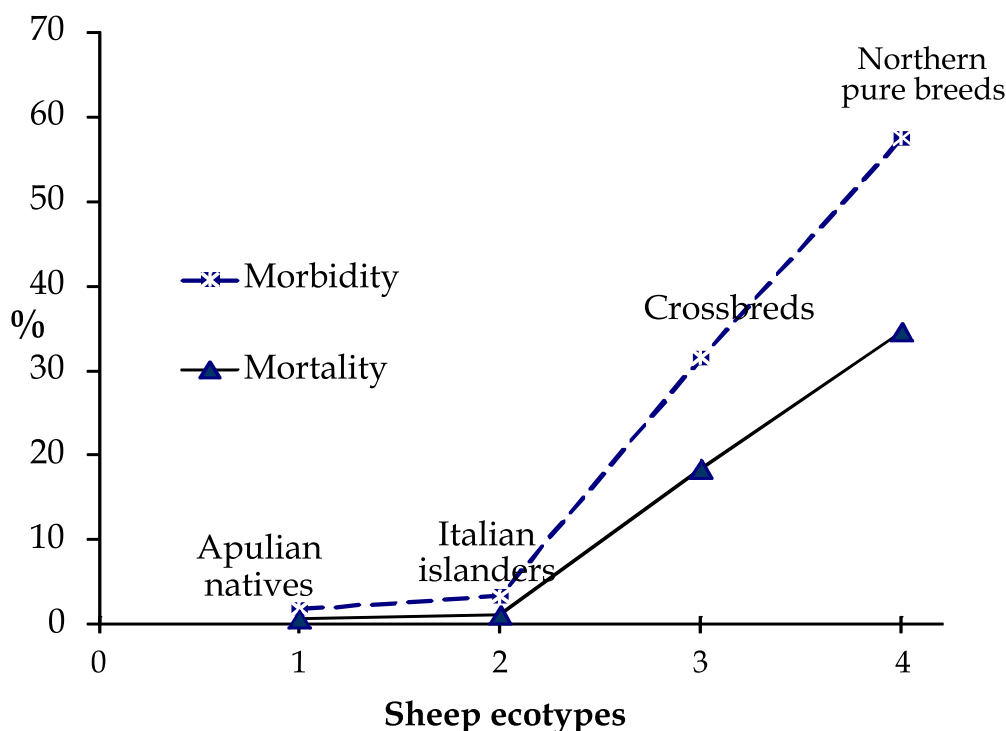
Fig. 2. Graphical representation obtained by processing morbidity and mortality data evidencing the influence of genotype on tolerance to pyroplasmosis in sheep living in Apulia. 1, Apulian native breeds (Altamurana, Gentile di Puglia and Leccese); 2, Italian islander breeds (Comisana and Sardinian); 3, Crossbreds (Finnish x Altamurana, Finnish x Leccese, Friesian x Altamurana, Friesian x Leccese; Romanov x Altamurana, Romanov x Leccese); 4, Northern pure breeds (Finnish, Friesian and Romanov). Modified from Pieragostini & Petazzi (1999).

A further element to consider is that attempts to improve the productivity of Apulian breeds by crossing them with the above exotic breeds failed because of the high mortality in generations F1 and F2, almost solely due to TBD. Though the mortality rates in crossbred animals were lower than those registered in the respective parental pure breeds, the number of individuals killed by the impact with endoerythrocytic pathogens was in any case too high (Fig. 2).

Pathogens were not accurately classified since the study analyzed data from farm records in which the veterinarians' diagnosis at death, due to TBD, always mentioned pyroplasmosis. The cases, which we were able to observe, concerned five Romanov sheep and seven Suffolk (occasionally found in the course of time and seriously ill prior to our visit). Examination of the animals always revealed classic symptoms of babesiosis and this was confirmed once the blood samples taken at the same time were analyzed. The haematological situation showed severe microcytic and hypochromic anaemia and *Babesia ovis (B. ovis)* was consistently identified in the blood smears.

By contrast, among the resilient breeds of sheep, the animals infected with pyroplasmosis showed only a state of discomfort which usually does not last more than few days and is characterized by a brief rise in temperature, slight dejection in the form of a tendency to move away from the flock, loss of appetite which might also be very transitory, translucent mucosae, slightly blueish against a pale background and in a few cases subicteric.

### 3.3 Piroplasmosis in naturally infected tolerant sheep

Resistance is a dynamic process of parasite regulation by the host. The pathogen must penetrate host cell barriers in sufficient numbers, attack target cells and replicate. Sub-clinical or clinical expression of the disease is dependent on the pathogen's virulence and the interaction between pathogen and host characteristics. Particularly, the phenomenon of tolerance to tick borne pathogens (TBP) is closely linked to a particular type of anaemia which is generally the symptom *par excellence* of the disease. In the tolerant animals, as shown in a study carried out on Altamurana sheep, this takes a benign macrocytic and hyperchromic form. A comparison of the haematological parameters of healthy sheep with those of sick sheep in table 4 showed that the latter presented a numerical deficiency of red blood cells that was compensated by the fact that the mean corpuscular volume (MCV) increased by about 50% as did the mean corpuscular haemoglobin (MCH). The results shown in table 4 did not stem from a dedicated investigation because the haematologic alterations were met with by chance when investigating on the functional effect of a rare alpha globin gene variant. At sampling, the affected sheep did not show any patent signs of the disease and thus only the haemocromocytometric parameters, the related observation of blood films and the results of the osmotic fragility test led to classifying the sampled animals in healthy and affected. Observation of blood films in this study and in other subsequent occasional analyses on Apulian sheep in similar conditions, highlighted that in most cases there were mixed infections in which *Anplasma* spp. and/or *Theileria* spp. and/or *Babesia* spp. occurred at the same time (Fig. 3). *B. ovis* was consistently present in the blood films of the affected animals with visible symptoms of haematuria. This fact, taken together with the evidence from tests on Romanov sheep infected and killed by babesiosis, convinced us that *B. ovis* was one of the causes of the pathogenetic activity in Apulian sheep, and certainly in non-native breeds. However, diseases often occur in clusters of time (years, seasons, production cycles, etc.) and space (herd, pasture, farm, region, etc.) and the prevalence of this pathogen was never the target of a dedicated epidemiological investigation.
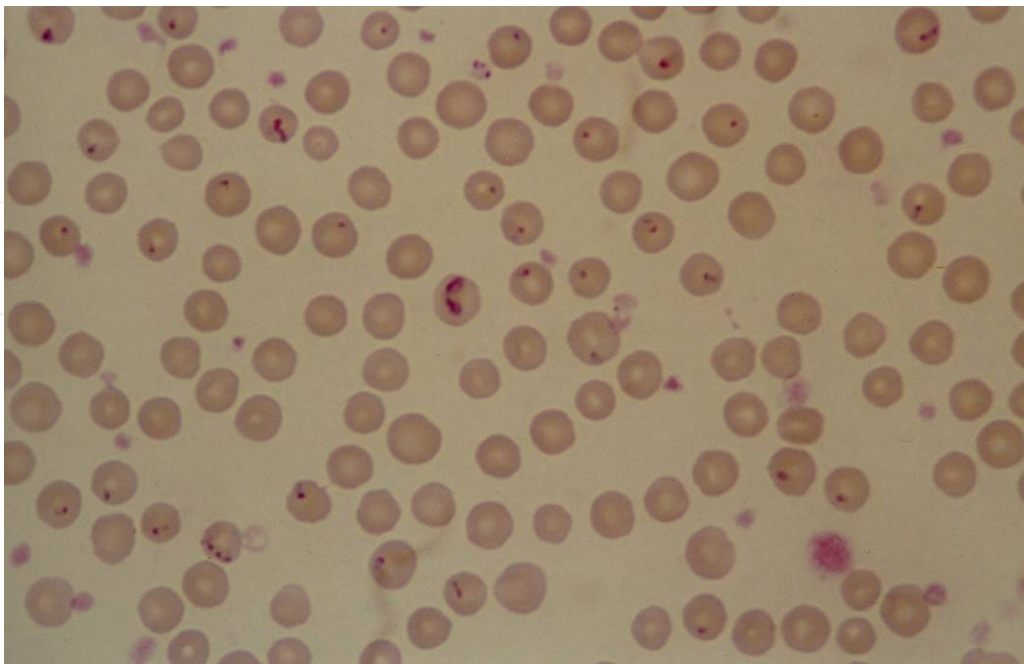


Fig. 3. Blood film showing a mixed infection of *Anaplasma* spp. and *Babesia ovis*.

| Haematological parameters | Samples | | Significance |
|---|---|---|---|
| | Healthy (N =22) | Affected (N =28) | |
| RBC ($10^6/\mu l$) | 9.5±0.19 | 6.3±0.70 | ** |
| Hb (g/dl) | 9.9±0.18 | 9.2±0.50 | * |
| PCV (g/dl) | 32.3±0.55 | 30.3±0.50 | ** |
| MCV (fl) | 34.3±1.43 | 48.7±1.29 | ** |
| MCH (pg) | 10.4±0.46 | 15.2±0.42 | ** |
| MCHC (g/dl) | 30.5±0.44 | 31.2±0.40 | n.s. |
| WBC ($10^3/\mu l$) | 9.6±0.40 | 9.5±0.37 | n.s. |
| MCF (g%NaCl for 50% haemolysis) | 0.72±0.02 | 0.82±0.03 | ** |

Table 4. Comparison of the haematological parameters recorded in healthy and affected
Altamurana sheep (mean values ± standard errors). Modified from Pieragostini & Petazzi,
1999. (RBC=Red Blood Cells; Hb=Haemoglobin %; PCV= Packed Cell Volume; MCV= Mean
Corpuscular Volume; MCH=Mean Corpuscular Haemoglobin, MCHC=Mean Corpuscular
Haemoglobin Content; WBC=White Blood Cells; MCF=Mean Corpuscolar Fragility)
*P<0.05;**P<0.01; n.s.= not significant.

### 3.4 Anaplasmosis in naturally infected splenectomized sheep

Anaplasmosis is one of the most important tick-borne diseases of ruminants worldwide. The
disease is caused by infection of animals with the obligate intraerythrocytic bacteria
*Anaplasma* spp. which is classified in the family *Anaplasmataceae*, order *Rickettsiales* (Dumler
et al, 2001). This section includes some experiences with sheep splenectomy and describes
disease onset and course in eight splenectomized TBD-tolerant sheep that were naturally
infected with piroplasms. Though the trials had been performed in different time periods,
the results obtained were very similar and the facts surrounding the experiments gave us
both general and specific insights into the field of splenectomy of carrier sheep from areas
where endoerythrocytic parasites are endemic.
Particularly in the first trial, the surgical operation had two purposes: a) to evaluate the rôle of
the spleen as a filter-pad to check parasites and as modulator of the direct response to anemia;
b) to obtain a high number of parasites in the blood to prepare a local specific antigen.
The following trials were mainly related to the need to obtain *A. ovis* which was isolated
from splenectomized sheep allowed to be naturally infected pasturing in tick areas.
Splenectomy was slightly traumatic for all the subjects and 24 hours after the surgical
operation the sheep showed normal functions. The sheep were identified with female names
for easier checking. Clinical evaluation was done on a daily basis and rectal temperatures
were recorded every morning for 12 weeks post splenectomy. Blood and serum samples
were routinely collected twice a week during the observation period. Haematological
variables were evaluated using a haematology analyzer. The erythrocyte fragility test was
performed by exposing erythrocytes to hypotonic saline solutions decreasing by 0.02%
starting from 0.86%. Parasites in the blood were checked by Giemsa staining every 3 days.
During the acute phase of the disease, the most important haematological values,
erythrocyte fragility and parasitaemia were monitored daily. In the case of Gilda, Lina and
Zoppina, which were part of the experiment to check the response to *A. ovis* infection of

different sheep breeds, described in section 5, parasite density was estimated on thin blood film and expressed as the percentage of parasitized red blood cells.

Fifteen days after the splenectomy, the general situation worsened and the animals became anorexic, staggering with a severe anaemia and dehydration. At the same time the RBC, Hb% and PCV values dropped (Tab. 5), and a number of organisms started appearing in the blood films (Rosalba showed a carpet of *A. ovis*; Stella a great deal of *A. ovis*; Lisa and Lola a great deal of *A. ovis* and a few *Babesia* spp.; Claretta a great number of *Theileria* spp.). Rosalba and Stella died of severe anaemia respectively 24 hours and 4 days after the diagnosis despite specific drugs and whole blood trasfusions with blood drawn from a donor subject. Claretta, Lisa and Lola showed less violent initial symptoms, the anaemic crisis was less severe and following a therapy with anti-protozoal drugs associated with desametazone they gradually began to eat and became clinically and haematologically healthy in 15-20 days. Since Lina, Zoppina, and Gilda, were included in the above cited experimental design to investigate the tolerance to *A. ovis*, they were constantly monitored and parasitaemia was recorded every two days after splenectomy. The cases of Lina and Zoppina allowed comparison between a mixed infection by *T. ovis* and *A. ovis* and an almost single infection by *A. ovis*. Interestingly, the two sheep coped differently with the infections. Though both animals were positive for *A. ovis* and *T. ovis* after splenectomy, the maximum of parasitized erythrocytes (MPE) by *T. ovis* peaked to 17% in Zoppina, while in Lina *T. ovis* caused a latent infection. Conversely, MPE by *A. ovis* in Zoppina was less than a half that of Lina (Tab. 5).

| | Splenectomized sheep | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Rosalba | Stella | Lisa | Lola | Claretta | Lina | Zoppina | Gilda |
| Year of splenectomy | 1994 | 1994 | 1994 | 1994 | 1994 | 2009 | 2009 | 2010 |
| Incubation Time (days)* | 19 | 21 | 25 | 32 | 42 | 29 | 35 | 21 |
| Max Temperature (C°) | 39.80 | 39.80 | 39.60 | 39.40 | 39.20 | 39.40 | 39.20 | 39.60 |
| Min PCV (g/dl) | 7 | 7 | 10 | 10 | 11 | 10 | 11 | 10 |
| PCV reduction (%) | 75 | 74 | 56 | 55 | 57 | 61 | 75 | 60 |
| Hb reduction (%) | 73 | 74 | 58 | 53 | 52 | 55 | 72 | 54 |
| Max parasitemia *A.ovis*(%) | >70 | >60 | n.e. | n.e. | n.e. | 36 | 15 | 60 |
| Max parasitemia *T.ovis* (%) | n.e. | n.e. | n.e. | n.e. | n.e. | 3 | 17 | 2 |

Table 5. Summary of clinical findings recorded in eight sheep splenectomised in different time periods (n.e.=observed but not estimated; *Incubation Time=number of days from first observation of infected blood cells on stained blood smears to the peak of the disease).

Then, Lina developed the disease after an incubation period of 29 days and recovered within a month, exhibiting a slight decrease in PCV (less than 25%) on post-splenectomy day 90 due to a slight increase in parasitaemia by *A. ovis* (Fig. 4). The two sheep were transfused with blood from a healthy donor sheep and treated every two days for a week with oxytetracycline (Terramicina long acting 1000 mg) and dexamethasone (Desashock Fortdodge Animal Health S.p.a., 80mg single dose). Both Lina and Zoppina quickly recovered from the disease, reaching normal blood values within four weeks, but, one month after their recovery, they had a relapse which they coped with successfully.
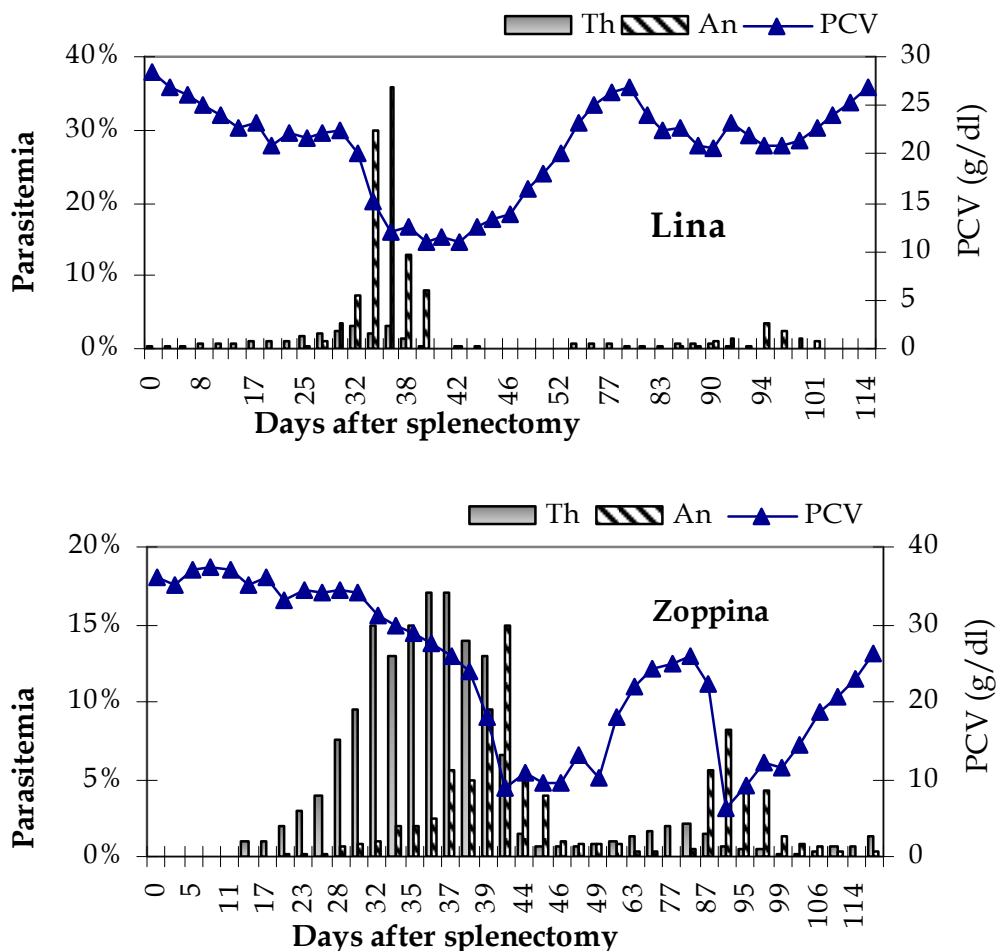
Fig. 4. Trend of PCV values and parasite densities (*An=Anaplasma ovis* and *Th=Theileria ovis*) expressed as percentage of red blood cells parasitized in Lina and Zoppina, the two splenectomized sheep monitored over a five week period after splenectomy.

During the recovery period, clinical examination revealed only pale mucous membranes. The pattern of evolution of the disease and recovery in Gilda was almost the same as in Lina except for a higher level of parasitaemia. In this instance *A. ovis* was the sole agent of the illness of our sheep. Secondly, the increase in *A. ovis* is apparently slowed down by the presence of *T. ovis* which seems to have a 'buffer effect' on the appearance of anaplasmosis but not in tempering its severity. Three points emerge from these results: i) in contrast to babesiosis and to the literature of some years ago (Radostits et al., 2000), hemoglobinuria did not occur in any of the seven severe cases of anaplasmosis; ii) treatment produced an immediate reduction in parasitaemia without leading to a complete clearance of the parasites; iii) disease relapse might be accounted for by the persistence of *A. ovis.*

Though not a novelty, in areas with enzootic erythrocytic parasitoses, even apparently healthy breeding animals may host pathogens and show tolerance and/or premunition to them without presenting with circulating parasites. This is a limit that should always be taken into account and may constitute a complication for any stress-associated situation. Anaemia secondary to anaplasmosis may evolve in a remarkably violent fashion probably due to the mechanism effected by the reticulo-endothelial system (RES) virtually with no

haemolysis. In our small experience the use of desametazone had a beneficial effect as it reduced the general response to the stimulation of the pathogen and particularly macrophage activity and improved red blood cell membrane response. Several years have elapsed since the first experiment and Lola, Lisa and Claretta got back to 'normal life' and, before their death, caused by old age, they showed no signs of disease which might have been related to haematological parasitosis. As to Lina, Gilda and Zoppina, they are back in the flock following a normal breeding and reproductive cycle. With no doubt the spleen naturally acts as an immunologically active filter-pad countering even severe red blood cell deprivation; its activity is particularly prominent in the presence of antibodies given that even after splenectomy these animals were still able to resist local diseases. There are grounds to believe that the animals may have a genetically derived tolerance to such instances based on active, diffuse and efficient structural systems which do not relate to one sole organ.

## 4. Response to experimental anaemia

It is difficult to distinguish whether, in the case of native sheep, the slightness of the degree of anaemia should be considered the cause or the effect of tolerance. However, it is certain that these animals have an unquestionable ability to maintain a good level of homeostasis during TBD  evidenced from the data shown in table 4, particularly those concerning PCV, Hb and MCHC.

To the purpose, four sheep belonging to a sensitive and non tolerant breed (Romanov), and four sheep to a sensitive but tolerant breed (Altamurana) underwent regular bleeding for seven days, stopping when the decrease of the packed cell volume ranged from 35 to 40%, the same as usually observed in clinical ovine babesiosis caused by *B. ovis* (Yeruham et al., 1998).

Over time the quantity and quality of the evolution of the haematological response were checked. The regression analyses performed to compare the two breeds with respect to the various data sets, gave the following results (Tab. 6):

- the intrabreed correlation coefficients recorded for PCV, Hb and RBC, were statistically significant only in the case of Romanov sheep, testifying to high difformity in the anaemization response between Romanov individuals, while Altamurana sheep behaved almost the same;
- the comparisons between the correlation coefficients obtained for PCV, Hb and RBC, in the two different breed groups were highly statistically significant.

Of these two points, while the latter might have been expected as the trial was based on the assumption of difference between the two breeds, the former result opens new vistas in the evaluation of the phenomenon. The low variability in the response to the anaemization exhibited by Altamurana sheep might be the result of the selection pressure acted by the constant presence of anaemizing parasites. Conversely the variability of the Romanov sheep could be taken as the individual response to the impact of an unusual stress.

As a general consideration, the two groups were composed by animals which were profoundly different and constantly on different levels from the haematological point of view. Both situations observed seemed to represent different aspects of normality, particularly the Altamurana sheep are constantly "poor" in the absolute levels of PCV, Hb and RBC and constantly "richer" regarding the derived parameters, MCV, MCH, MCHC, the latter being constantly those expressing haematological "efficiency" in the face of

anaemia (Pieragostini & Petazzi, 1999). So if it is a matter of fact that from the numerical point of view, the two breeds' responses to anaemization are to a large extent not very dissimilar, the greater efficiency of the local breeds is beyond doubt. It is not to be excluded that this may be identified in their greater capacity to cope with anoxemic stress, both by the production of red globules enriched with haemoglobin and maybe also by accelerating the turnover of older and less efficient red blood cells.

| Contrasts | Haematological parameters | | |
|---|---|---|---|
| | RBC | Hb | PCV |
| Within Altamurana | n.s. | n.s. | n.s. |
| Within Romanov | *** | *** | *** |
| Between Altamurana and Romanov | **** | **** | **** |

Table 6. Statistical significance of the differences between the correlation coefficients calculated by the regression analysis performed to compare the two breeds, Altamurana and Romanov, with respect to the hematological parameters RBC (Red Blood Cells), Hb (Haemoglobin) and PCV (Packed Cell Volume). ***P<0.001; ****P<0.0001; n.s., not significant.

The reading of these haematological aspects should be looked at without losing sight of the general aspect of the overall comparison between the two breeds. From this point of view, the considerable difference in absolute values, apparently almost negligible as regards the curve trends, becomes very striking in the comparison between the general situations of overall well-being of the two breeds compared. The Altamurana sheep continued to exhibit apparent good health when subjected to anaemization, at ease with their surroundings, ready to feed and drink. Conversely, the Romanovs exhibited a serious dulling of the senses and lack of reaction once anaemization was achieved; this necessitated support treatments with rehydrating solutions to allow them to overcome their state of anergy (Pieragostini & Petazzi, 1999). These results strongly support the hypothesis that, beyond the environmental factors such as stress, nutrition and other conditions, which in general facilitate infections (Agyemang et al., 1990; Bennison et al., 1998; Oppliger et al., 1998) and which are supposed to be particularly relevant in the case of non-native breeds, genetic predisposition plays a major role also in the pathogenesis of TBD.

## 5. Response to *Anaplasma ovis* infection in experimentally infected sheep

Animal well-being has become a significant concern among consumers who expect food animals to be well treated, raised in idyllic environments, and free of disease. Consumers also expect their meat products to be free of residual antibiotics and therapeutic drugs. For these reasons, new approaches or alternatives to addressing animal diseases are needed. One approach is genetic selection for animals resistant to disease, that is: an approach whose focus is on accepting certain constraints of the environment and using breeds that can cope with these constraints, as opposed to the earlier approach which focussed on changing the environment to create opportunities for exotic breeds to be productive. But identifying the phenotype for disease resistance is difficult.

As to TBD, the response is known to be under multi-factorial regulation (Horin, 1998; Aguilar-Delfin et al., 2001). As highlighted in the above section 4, the phenomenon of tolerance is a broad-based one and possibly not unrelated to the erythropoietic system in different sheep breeds or to the haemoglobin genetic systems (Pieragostini et al., 2003; Pieragostini et al., 2006).

Anyway, the success of selection for disease resistance is dependent on correctly identifying the disease agent and the phenotype for disease resistance. For example, as to TBP in small ruminants, there are several reports concerning the presence of *Babesia, Theileria,* and *Anaplasma* species infecting sheep and goats in many countries world-wide but, in many regions of the Old and the New World, the identity of the tick-borne disease agents of sheep and goats and of their vector ticks is uncertain. But perhaps, the biggest challenge of selecting for disease resistance is to accurately identify the phenotype for disease resistance and/or to have reliable genetic markers with high predictive values for a disease phenotype. Phenotypic variability induced by parasites is a matter of fact, as impressively exemplified by the high number of haemoglobinopathies in human populations living in malaria-endemic areas (Evans & Wellems, 2002).

Recalling Feynman's[1] saying that nature repeats itself at every scale, we suggested that the unusual haemoglobin polymorphism recorded in Apulian native sheep breeds and the related functional effects might have an adaptive significance, also being somehow related to the selective pressure of tick borne parasites (TBP) (Pieragostini et al., 1994; Pieragostini et al., 2003; Pieragostini et al., 2006). Based on these considerations, we aimed to define the phenotype of the tick borne diseases in different sheep breeds starting from the one caused by *A. ovis*, the most common parasite in our area as confirmed by a small survey on sheep TBP performed in 10 farms (throughout Apulia) on 240 individuals. *A. ovis* was identified in 58% of samples, followed by *T. ovis* (5.8%) and *T. annulata* (4.5%). *Theileria* spp. were present in mixed infections with *A. ovis, B. ovis* (0.9 %) or *Babesia* spp. (0.9 %). In particular the presence of *A. ovis* was confirmed by specific polymerase chain reactions (PCRs) for *Anaplasma* spp. (Stuen et al., 2003) and *A. ovis* (de la Fuente et al., 2005; de la Fuente et al., 2007). Then PCRs followed by reverse line blot hybridization of the amplified 18SrRNA gene from *Theileria* and *Babesia* species, was used to detect specific probes for *Theileria/Babesia catch all, Theileria sp1 china, Theileria sp2 chinal, T. buffely, T. annulata, T. velifera, T. taurotragi, T. mutans, T. lestoquardi, T. ovis, B. bovis, B. bigemina, B. crassa, B. motasi, B. ovis, B. major, B. divergens, T. hirci, B. sp1 (Turchey), B. sp2 (Lintan*) (Schnittger et al., 2004).

| Year | Step 1 | Year | Step 2 |
|---|---|---|---|
| 2009 | Search for carriers | 2010 | Search for carriers |
| 2009 | Splenectomization | 2010 | Splenectomization |
| 2009 | Infection of 8 Suffolk and 8 Comisana characterized by normal alpha globin gene arrangements and different beta genotypes | 2010 | Infection of 18 Altamurana characterized by different alpha globin gene arrangements |

Table 7. Experimental design.

[1] Richard Phillips Feynman (May 11, 1918 – February 15, 1988) was an American physicist who received the Nobel Prize in Physics in 1965.

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Thus, a project was set up to evaluate the response to anaplasmosis in susceptible and tolerant sheep breeds including the use of haemoglobin genetic systems as genetical markers of tolerance to the disease. Summarized actions are described in table 7.

## 5.1 Materials and methods
### 5.1.1 Search for carriers and parasites
Sixty ewes were sampled from a flock extensively reared in the countryside near Bari. The flock consisted of approximately 250 heterogeneous subjects belonging mainly to TBD tolerant breeds and crossbreds. The presence of TBPs was checked in the blood samples by a PCR-based molecular approach as described above. Most of the sampled animals were found to carry *A. ovis* mixed with other TBPs. Based on the results of the flock survey and the consent given by the breeder, three ewes carrying *A. ovis* and *T. ovis* were selected and purchased. Following the experimental design (table 7) Lina and Zoppina were splenectomized in 2009 while Gilda in 2010, as described in subsection 3.4.

### 5.1.2 Animals
Selected animals 7/8 months of age were involved in this study. Lambs less than six months of age were purchased and housed at the Medical Clinics of the Faculty of Veterinary Medicine of the University of Bary. Upon arrival at the Faculty of Veterinary Medicine, the animals were weighed and faecal samples were obtained to establish their worm burdens. Feet were checked for foot rot. The animals were dewormed with a broad spectrum anthelmintic. All of them were then housed in a tick proof isolation unit. In particular, in 2009 the lambs were selected based on different breed and equally divided between Suffolk and Comisana.
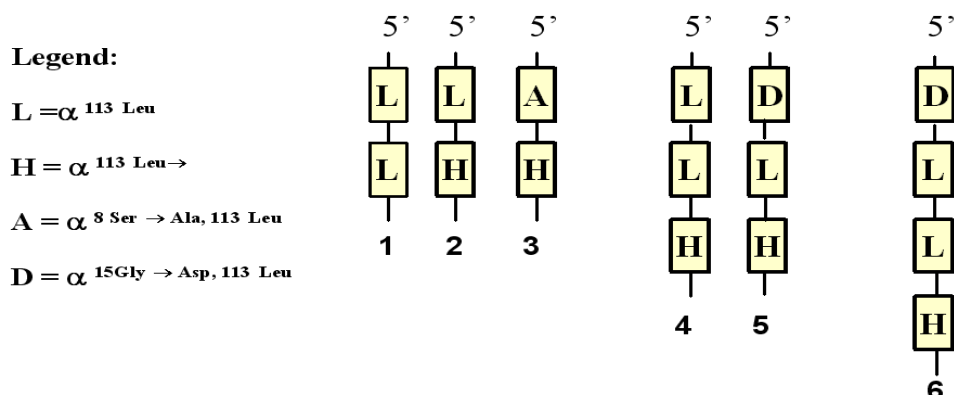


Fig. 5. Alpha-globin gene haplotypes detected so far in sheep, namely: haplotypes 1, 2 and 3 are normally duplicated (NH); haplotypes 4, 5 and 6 show extranumeral alpha gene arrangements (EH); particularly haplotypes 4 and 5 are triplicated while haplotype 6 is quadruplicated.

All the lambs were characterized by a normal duplicate alpha gene arrangement (Fig. 5) and most of them by homozigosity at the beta globin loci. Owing to high frequency of HBBA gene in the Suffolk breed, three out of the eight Suffolk lambs were HBBAB heterozygotes.
In 2010 eighteen Altamurana lambs less than six months of age, housed and treated as above described, were selected based on different alpha globin genetic arrangements. Nine lambs were homozygotes for the normal duplicate alpha gene haplotype (NH), the others carrying an extra-numeral alpha haplotype (EH) (Fig.5); most of the 18 lambs were homozygotes for the HBBB allele at the beta globin loci.

### 5.1.3 Experimental infection

*A. ovis* was isolated from one of the above splenectomized sheep. Lina was the donor for the 2009 lambs and Gilda for the 2010 lambs. Parasite density was estimated on thin blood film obtained by the buffy coat method and expressed as the percentage of parasitized red blood cells. At the peak of parasitaemia in the donor sheep (36% and 60% of red blood cells parasitized respectively), about 400 ml of blood were obtained and each lamb in the breed groups was inoculated intraperitoneally with 25 ml of infected blood.

### 5.1.4 Clinical observations

Clinical evaluation was done on a daily basis and rectal temperatures were recorded every morning for 8 weeks post infection. Blood and serum samples were collected twice a week during the observation period. Haematological variables were evaluated using a haematology analyzer. Parasite density was estimated on thin blood film as above described.

### 5.1.5 Haemoglobin phenotype

The reversible switch from haemoglobin A to C was observed in the above HBBAB Suffolk lambs. The expression of the silent gene encoding for Hb C was detected by isoelectric focusing and quantified by high performance liquid chromatography (Alloggio *et al.*, 2009).

### 5.1.6 Statistics

First, differences between breed groups for clinical and haematological data were assessed using analysis of variance (ANOVA) by GLM procedure (SAS, 1990). A second ANOVA was carried out only for the Altamurana group, considering the interaction between the alpha globin type (2 levels: NH and EH) and each clinical variable. The last ANOVA was carried out only for the Suffolk group considering the interaction with the beta globin type (2 levels: AB and BB) of the linear and quadratic regression of each haematological variable on the number of days from the infection, with three of the Suffolk that were AB compared to as many BB. This analysis was performed for the Suffolk, where, as cited above, both AB and BB genotypes were found, whereas only BB animals occurred in the Comisana.

## 5.2 Results and discussion

The following is an overall picture of the findings where they are reported and discussed relating to the different approaches.

### 5.2.1 Clinical findings

Host responses in the three experimentally infected sheep groups were first compared mainly according to typical high fever periods, microscopic observation and haematological values. *A. ovis* began to appear in the blood a week before the fever and the following records showed that the maximum of erythrocytes parasitized by *A. ovis* in any case did not exceed 2%.

All the animals developed the disease (Table 8) but symptoms varied in terms of severity and duration and none died. Fever syndrome (listlessness, anorexia, weakness, ruminal stasis, respiratory distress, increased heart and respiratory rates) and pallor of the mucous membranes were recorded in seven of the Suffolk group, in only one of the Comisana group and in none of the Altamurana.

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| Breed | Dose of infection | Symptoms | Need for therapeutic intervention | Morbidity | Expected Mortality |
|---|---|---|---|---|---|
| Suffolk | 36% | very severe | 7 out of 8 subjects | 100% | 87.5% |
| Comisana | 36% | severe | 1 out of 8 subjects | 100% | 12.5% |
| Altamurana | 56% | mild | none | 100% | 0% |

Table 8. Overview of responses to anaplasmosis in the three analyzed breeds.

The haematological patterns were then analyzed in detail comparing the intra breed variations between the different physiopathological moments – normal health status (time $0=T_0$), acute phase (time $1=T_1$ ) recovery phase (time $2=T_2$ ) - and the between breed variations intra physiopathological moments (table 9). Finally, clinical parameters, such as incubation time (I.T) after infection, temperature peak (T.P.), percentage decrease in haematocrit ($\Delta$ HCT), percentage decrease in haemoglobin content ($\Delta$ Hb) expressed as gr Hb/dl blood, percentage decrease in red blood cells ($\Delta$ RBC) were evaluated for each breed  (Table 10).

| Breed | Parameter | $T_0$ | | | $T_1$ | | | $T_2$ | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | SD | | Mean | SD | | Mean | SD | |
| | PCV (g/dl) | 31.9 | ± 2.8 | a | 12.7 | ± 2.7 | A | 23.9 | ± 2.3 | a |
| | Hb (g/dl) | 11.5 | ± 1.0 | a | 4.7 | ± 0.7 | A | 7.7 | ± 0.7 | a |
| | RBC ($10^6$/µl) | 12.5 | ± 1.2 | A | 4.5 | ± 0.8 | a | 7.2 | ± 1.0 | |
| Suffolk | MCV (fl) | 25.0 | ± 1.0 | A | 32.3 | ± 1.6 | A | 33.3 | ± 3.0 | A |
| | MCH (pg) | 9.2 | ± 0.3 | B | 10.5 | ± 0.6 | A | 10.7 | ± 0.6 | A |
| | MCHC (g/dl) | 36.8 | ± 1.0 | A | 32.5 | ± 1.2 | | 32.3 | ± 0.8 | |
| | WBC ($10^3$/µl) | 8.6 | ± 1.1 | | 10.9 | ± 2.4 | | 0.3 | ± 1.1 | a |
| | PCV (g/dl) | 35.0 | ± 2.4 | b | 11.3 | ± 2.7 | A | 26.2 | ± 1.7 | b |
| | Hb (g/dl) | 12.6 | ± 1.0 | b | 4.7 | ± 0.5 | A | 8.6 | ± 0.7 | b |
| | RBC ($10^6$/µl) | 11.9 | ± 1.3 | A | 5.1 | ± 0.8 | a | 6.9 | ± 0.7 | |
| Comisana | MCV (fl) | 29.7 | ± 2.3 | B | 29.1 | ± 3.2 | B | 38.4 | ± 3.2 | B |
| | MCH (pg) | 10.7 | ± 0.7 | A | 9.4 | ± 1.1 | B | 12.5 | ± 0.9 | B |
| | MCHC (g/dl) | 35.9 | ± 1.3 | A | 32.1 | ± 0.4 | | 32.5 | ± 1.0 | |
| | WBC ($10^3$/µl) | 10.1 | ± 2.7 | | 7.5 | ± 1.4 | | 10.4 | ± 2.9 | b |
| | PCV (g/dl) | 31.2 | ± 3.2 | a | 21.6 | ± 3.1 | B | 25.8 | ± 2.4 | b |
| | Hb (g/dl) | 0.4 | ± 1.0 | C | 7.1 | ± 1.0 | B | 8.2 | ± 0.8 | b |
| | RBC ($10^6$/µl) | 9.4 | ± 1.0 | B | 6.2 | ± 1.0 | b | 7.1 | ± 0.8 | |
| Altamurana | MCV (fl) | 33.2 | ± 1.9 | C | 34.7 | ± 1.7 | C | 36.6 | ± 2.0 | B |
| | MCH (pg) | 10.6 | ± 0.5 | A | 11.4 | ± 0.5 | C | 11.6 | ± 0.5 | B |
| | MCHC (g/dl) | 31.7 | ± 0.7 | B | 32.8 | ± 0.9 | | 31.9 | ± 1.0 | b |
| | WBC ($10^3$/µl) | 10.1 | ± 2.6 | | 9.6 | ± 2.0 | | 10.1 | ± 1.4 | B |

Table 9. Haematological parameters assessed for the three analyzed breeds, namely  normal health status before infection (time $0=T_0$), during the acute phase (time $1=T_1$ ) and during the recovery phase (time $2=T_2$ ). Means within columns with different letters significantly differ: capital letters: P <0.01; small letters: P<0.05.

As already reported, none of the animals had more than 2% erythrocytes parasitized by *A. ovis*. This confirms the role the spleen plays in the phagocytosis and clearance of parasitized erythrocytes; otherwise only splenectomized sheep showed significant percentages of parasitized erythrocytes (table 5).

Mean parasitaemia in the single group could theoretically be inferred from the Δ RBC and conclude that the higher the Δ RBC value, the higher the suceptibility of the erythrocytes to *Anaplasma* infection. According to the results shown in table 8 depicting the response of the three breeds to infection, broken down according to the dose of infection, symptoms, need for therapeutic intervention and expected mortality, seven out of the Suffolk group recovered after being treated every two days for a week with oxytetracycline and dexamethasone whereas seven subjects of the Comisana group recovered from clinical anaplasmosis with no drug treatment other than a single dose of dexamethasone. The highest degree of tolerance was observed in the Altamurana group where all the subjects showed only mild alteration of behaviour and basic life functions. Comparison of the haematological patterns of the three breeds at $T_0$ in table 10 revealed that, in normal health conditions, the differences which may be noticed are consistent with those of earlier studies described in section 2, indicating that both environmental and productive specialization seem to account for the different physiological results.

Hence the Altamurana breed is characterized by significantly lower RBC and Hb values and by significantly higher MCV, MCH and MCHC values than Suffolk, a northern meat breed, while Comisana, a Mediteranean dairy breed has intermediate values. At $T_1$ and $T_2$ the same variation pattern may be observed, that is:

* haematologically, *A. ovis* infection does not seem to seriously affect Altamurana whose response may be described as a moderate normochromic normocytic anemia followed by a normochromic macrocytic pattern representing an active regeneration phase.
* conversely, the Suffolk and Comisana animals exhibited a violent response to *A. ovis* with a severe anaemia. The hyperchromic and macrocytic anaemia in the Suffolk was followed by a slow regeneration and the hypochromic normocytic anaemia of the Comisana by an active regeneration phase, similar to the Altamurana pattern, as documented by the high MCV values.

The results shown in table 10 confirmed the differences among the three breeds both in terms of quantitative (Altamurana *vs* Suffolk) and temporal (Comisana *vs* Suffolk) variation in haematological parameters. While Suffolk animals displayed the most severe reduction in the number of erythrocytes and haemoglobin content, Altamurana was characterized by a more controlled response, with only a minor and more gradual decline in RBC and Hb values. Comisana experienced a more severe reduction in the number of erythrocytes than did Suffolk, though the decline in RBC values was not accompanied by a decrease in the total haemoglobin content as severe as that observed in Suffolk. This could be due to the significantly higher MCH values characterizing the Comisana haematological pattern as compared to the Suffolk.

Considering the overall responses shown in table 8 and detailed in table 9 and 10, there is no doubt that we are dealing with very different animal groups exhibiting different physiopathological phenotypes where a healthy haematological picture plays a relevant role.

| Breed | I.T (days) | T.P. (°C) | ΔHCT (%) | ΔHb (%) | ΔRBC (%) |
|---|---|---|---|---|---|
| Suffolk | 24.2[A] | 40.5 [A] | 65.4[A] | 60.2 [A] | 60.3 [A] |
| Comisana | 38.8[B] | 39.9 [B] | 57.7 [B] | 56.9[A] | 55.9[A] |
| Altamurana | 25.3[A] | 39.9 [B] | 19.2 [C] | 19.8 [B] | 33.7 [B] |

Table 10. Clinical parameters assessed for the three breeds analyzed at the peak of the disease, namely incubation time (I.T) after infection, temperature peak (T.P.), percentage decrease in haematocrit (ΔHCT), percentage decrease in haemoglobin content (ΔHb), percentage decrease in red blood cells (ΔRBC).

### 5.2.2 Functional effect of beta globin genes on the recovery from anemia

As to the functional effect of beta globin genes on the recovery from anemia, Figure 6 shows the results of the analysis performed in the Suffolk, where both AB and BB genotypes were found.
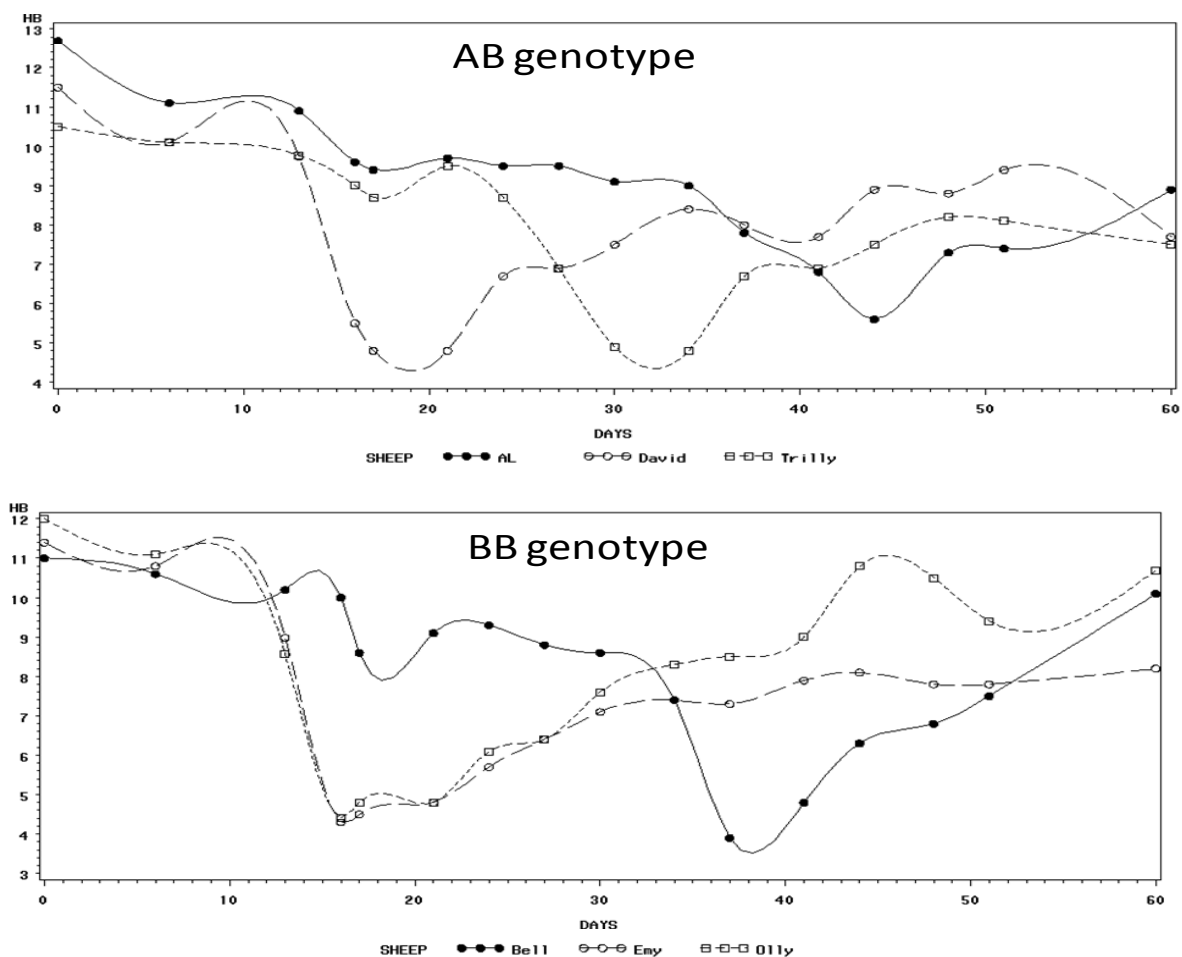


Fig. 6. Trend of Haemoglobin (Hb, g/dl) as a function of the number of days from the infection (DAYS) for the haemoglobin genotypes AB and BB (Modified from Alloggio et al. 2009).

Goats and some sheep under conditions of erythropoietic stress (anaemia) or hypoxia, synthesize a juvenile haemoglobin (Hb) type, Hb C, where β-globin is encoded by the silent gene *HBBC*. Anaemia causes a change in the type of circulating haemoglobin only in sheep carrying βA-globin haplotype, where Hb A is replaced by Hb C. Pioneered by the work of van Vliet and Huismam (1964), the Hb C in Caprini species has been thoroughly studied and particularly the mechanism of reversible switching has triggered focused research in the 70's (Nienhuis and Anderson, 1972; Nienhuis and Bunn, 1974). Little information is available on the effect of Hb A replacement by Hb C. Owing to the high oxygen affinity of Hb C (Huisman and Kitchens, 1968), the reversible switch from Hb A to Hb C may be considered a way to cope with the reduced amount of oxygen available at higher altitudes and thus suggest a positive effect on the fitness of mountain Caprini. Conversely, in the case of erythropoietic stress, it was suggested (Pieragostini et al., 1994; Pieragostini et al., 2006) that Hb C might negatively affect peripheral oxygen delivery and worsen the clinical picture of sheep breeds native of areas with endemic haemotropic pathogens. Hence, in the present case, the *conditio sine qua non* for checking the effect of beta globin genotype was the detection of Hb C switched on in AB individuals following a strong erythropoietic stress. As an example, figure 6 also shows the different trend after infection (days = 0) of the Hb content for the AB and BB ewes. Apart from individual differences observed in reaching the lowest Hb values, the recovery was always faster in BB sheep, as also indicated by the significantly higher quadratic regression coefficient of Hb for BB *vs* AB genotype (0.0045 *vs* 0.0027, P < 0.03). The different behaviour of one of the three heterozygous subject (Figure 6) may be justified by its less severe haematological picture due to a lower anoxaemic stress, also confirmed by the lack of the Hb A to Hb C switch in the same animal.

### 5.2.3 Functional effect of alpha globin system on the response to the anaplasmosis

As mentioned before, the extra numeral alpha globin haplotypes (EH) were suggested to be related to the host's response to TBDs. The unusual frequency of EH recorded in southern Italian sheep breeds and the peculiar haematologic pattern exhibited by the EH homozygous subjects may be taken as evidence of a selective advantage of the corresponding phenotypes in endemic TBD areas. Individuals carrying extra alpha-globin genes exhibit an overall blood picture mimicking a thalassaemia-like syndrome. In greater detail, when the erythrocytes of EH homozygotes were compared with those of NH individuals, the former had fewer erythrocytes that were bigger in size and had a higher Hb content and a greater erythrocyte osmotic fragility. These changes in EH homozygotes were assumed to produce an unfavorable environment for the parasites.

Thus, the trial checked this hypothesis as the different haematological patterns and the accelerated turnover of erythrocytes of EH individuals compared to the NH ones were expected to produce differences in the spread of the pathogen into the host blood.

Unfortunately a relevant element of prejudicial questions to obtain maximum results was the fact that only EH heterozygotes were present in the Altamurana group, since no homozygous lambs were found during the population survey. In normal health conditions, such as those recorded in the experience reported by Pieragostini et al. (2003), the EH heterozygotes showed an intermediate pattern, between that of EH homozygotes and that of NH homozygotes (Pieragostini, unpublished data).

The second relevant element was the level of response which undoubtedly is a limiting factor in checking the results.

Thirdly, owing to experimental constraints, the sample size was of nine subjects per each alpha haplotype group. Hence, based on these considerations, we could not expect striking results as to the functional effect of the alpha globin gene arrangements, except in the case of a strong interaction with the response to experimental infections. Despite our hopes, our concerns were well-founded because all the above elements of prejudicial questions affected the results.

| α-globin gene arrangements | N | T.P. (°C) | PCV (g/dl) | Hb (g/dl) | RBC ($10^6/\mu l$) | MCV (fl) | MCH (pg) | MCHC (g/dl) | WBC ($10^3/\mu l$) |
|---|---|---|---|---|---|---|---|---|---|
| NH | 9 | 40.1 [A] ± 0.18 | 25.9 ± 1.55 | 8.1 ± 0.89 | 7.8 ± 0.85 | 33.1 ± 1.03 | 10.4 ± 0.51 | 31.4 ± 0.51 | 10.3 ± 1.29 |
| EH | 9 | 39.8 [B] ± 0.12 | 26.3 ± 1.18 | 8.5 ± 0.39 | 8.0 ± 0.31 | 33.7 ± 0.73 | 10.6 ± 0.43 | 31.4 ± 0.47 | 10.5 ± 1.17 |

Table 11. Temperature peak and haematological parameters assessed during the acute phase of the disease, for the two Altamurana groups classified on the basis of the α-globin gene arrangement. Means within columns with different letters significantly differ; capital letters: P <0.01.

Altamurana subjects exhibited a very mild symptoms and no patent differences could be recorded in terms of haematological pattern between the EH and NH individuals within the Altamurana group. The EH group had a temperature peak that was significantly lower (P<0.001) than that of the NH group. This suggested that the level of response to infection in the EH group was lighter than in the NH group (Table 12). Morever, as shown in table 11, though no significance was attained by the ANOVA when the mean values of the haematological parameters of the two groups were compared, a univocal trend emerged whereby the RBC, PCV and Hb values in the EH group decreased less than in the NH group. These two phenomena seem to indicate a milder *Anaplasma* infection in EH subjects than in the NH individuals.

## 6. Conclusions

Several examples of breed-related tolerance to diseases have been reported worldwide but often the claims made for specific breeds have not been subject to scientific investigation. As to small ruminants, only tolerance to Heartwater (Cowdriosis) has been documented for Damara, a South African native sheep breed (Commission on Genetic Resources for Food and Agriculture, 2007). This report extends our knowledge about tolerance to tick borne diseases. The main findings can be summarized in the following points:

- Tolerance to piroplasmosis is documented for Apulian (Altamurana, Gentile di Puglia and Leccese) and Italian islander (Comisana and Sarda) sheep breeds.
- Non-tolerance to piroplasmosis is documented for Finnish, Friesian and Romanov breeds.
- Suffolk breed is shown to be not tolerant to anaplasmosis.
- Different physiological pattern and environment of origin may explain breed-specific haematological characteristics.
- Altamurana sheep breed is tolerant to anaemia *per se.*
- The response of Altamurana to simultaneous *B. ovis* and *A. ovis* infection results in a mild anaemia.
- There seems to be confirmatory evidence that haemoglobin genetic systems underlie the host response in the acute phase of disease and in recovery.

## 7. Aknowledgements

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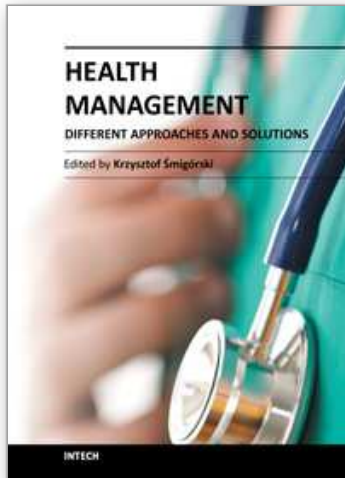
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