5. DISCUSSION

Ants *in tetania* were found mostly in the late spring, from the end of April until June, in keeping with the results of other studies conducted in the same area (SCHUSTER, 1991). This is related to the ecology and population structure of the intermediate hosts. Previous studies conducted in the same area (SCHUSTER, 1993) showed that the population structure of *Helicella obvia* was characterised by fluctuations, with small snails predominating from April to June, medium size snails from July to September, and the largest snails in the spring of the following year. The infection rate with sporocysts and snail age was compared and it was shown that young specimens of *H. obvia* in the first year were less involved in the epidemiology of dicrocoeliosis than the medium shell diameter snails, especially in the spring of the second year (SCHUSTER, 1993). The larger snails seem to be more susceptible to *D. dendriticum* because of their active metabolism that provide good nutritional conditions for developing sporocysts (ALUNDA and ROJO-VÀZQUEZ, 1983). During the summer of the second year a decrease of the infection rate was registered, due to the death of heavily infected snails in which sporocysts causes disruption of the hepatopancreas (important for glycogen storage), thus shortening of life expectancy (SCHUSTER, 1992). Therefore, the peak number of sporocysts is registered in April of the second year of the snail life cycle. Since the development of metacercariae in the ants take one-two months, the highest number of paralyzed ants is registered in May, as reported in the present study.

The mean prevalence of metacercariae infection in collected ants *in tetania* was 97.15%. This result is consistent with another (MANGA-GONZALEZ *et al*., 2001). In fact the brains of the dissected ants were not examined, so it cannot be ruled out that they contained only the brainworm. They could also have been wrongly collected among others in that stage, and it has to be considered that *tetania* could be caused by other factors, such as infection by the fungus *Entomophtora* spp. (LOOS-FRANK and ZIMMERMAN, 1976).

Also the metacercariae burden abundance is consistent with the results of studies conducted by Schuster, who found mean values of 76 metacercariae per ant and a burden range of 2-313 (SCHUSTER, 1991).

The establishment rates of *D. dendriticum* in sheep found in the present investigation (average 30% of infection dose, see Table 4) are similar to those reported by WOLFF (1984) (35% of infection dose).

As far as investigations on experimental serological tests are concerned, surveys on immune response dynamics of experimentally infected sheep with *D. dendriticum* (PIERGILI FIORETTI *et al*., 1980; GONZALEZ-LANZA *et al.*, 2000) are scarcely reported in the literature.

In the present study antibodies against both types of antigens were detected at 30 days after infection, one month before that coprological positivity was detected, in accordance with the result of other authors (GONZALEZ-LANZA *et al.*, 2000; MANGA-GONZALEZ and GONZALEZ-LANZA, 2005). No difference in timing of response was observed between low infected lambs and high infected lambs, in contrast with what noted by BODE and GEYER (1981) using experimentally infected hamsters.

The data obtained in this study showed that the pattern of IgG antibody responses assayed with E/S and So. antigens of *D. dendriticum* adults in sheep, did not substantially differ. These results agreed with similar studies (AMBROSI *et al.*, 1980; GONZALEZ-LANZA *et al.*, 2000). In experimentally infected sheep, GONZÁLEZ-LANZA showed that antibodies to *D. dendriticum* can be first detected by indirect ELISA 30 days after infection. In previous work (CAMPO *et al*., 2000), the average prepatent period was also 59 days after infection (49–79 days range). Also CALAMEL (1977), using the indirect immunofluorescence technique, found anti-*D. dendriticum* antibodies in sheep 6-13 weeks before the coprological diagnosis. LECLIPTEUX (1998) found out that the antigen competition assay was able to detect the presence of infection as soon as six days after the start of the experimental infection by *F. hepatica* in cattle. This confirms that serological diagnosis of dicrocoeliosis could be very useful because it could detect early infections, when juvenile flukes are still in the migratory phase (SANCHEZ-ANDRADE *et al*., 2003).

The antibody levels remained high during the whole period of study (150 days), in accordance with the result of other authors (GONZALEZ-LANZA *et al.*, 2000, SANCHEZ-ANDRADE *et al*., 2003, BODE and GEYER, 1981). This persistence of the antibody level had already been recorded by BODE and GEYER (1981) in golden hamsters and could be of great interest to sero-epidemiological studies.

In our study the correlation between OD values and parasitic burden could not be tested, because of the low number of experimentally infected lambs. Anyway also in other studies the results are controversial. GONZALEZ-LANZA (2000) showed no significant correlation between OD values and parasitic burdens. The number of parasites recovered from the infected lambs – less than 1000 worms – was not enough to produce differences in

antibody response. Nevertheless, other authors (BODE and GEYER, 1981) detected a highly significant correlation between the antibody titre developed later and the maximum value of parasite burden in animals with low and moderate infection, and, because of this, they considered ELISA the method of choice among other diagnostic tests (precipitation test, complement fixation test, indirect haemoagglutination test).

The specificity of the experimental ELISA test for *D. dendriticum* in relation to *Fasciola* spp. infection was tested using field samples from sheep coming from areas endemic for fasciolosis, and being coproscopically positive for *Fasciola* eggs. Cross-reactions with excretory/secretory (E/S) antigen were present in 45% of the samples, and 72% of samples cross-reacted against somatic (So.) antigen.

Using So. antigens, higher percentage of cross reactions with other trematodes sera was expected, because of the lower specificity of such protein extracts. In fact, the use of whole worm extracts as antigen can offer only low specificity and sensitivity for the serological diagnosis of *D. dendriticum* infections, as for some other helminthosis (GONZALEZ-LANZA, *et al.* 2000). Purification of fluke antigens or the use of parasite surface molecules as antigens could improve assay sensitivity.

Cross reactions were registered also with E/S antigen, although at a lower degree. Although cross reactions could be present, this could also be explained because the serum samples used are field samples from adult sheep, which might not be *Dicrocoelium*-free. Although coprologically negative for dicrocoeliosis, it must be kept in mind that, as mentioned in chapter 1, coprological examination is not a reliable diagnostic tools for dicrocoeliosis, thus it cannot be excluded that those animals are not or have not been previously infected with *D. dendriticum*.

Therefore, in order to assess test specificity, experimentally *Fasciola* infected animals are needed to prove cross reactions in standard conditions.

Cross infections with other nematodes were registered (*D. filaria*, *O. venulosum*, *O. ostertagi*, *T. colubriformis*, *C. curticei*, *N. battus*, *H. contortus*), but only with sera from repeatedly infected animals, whereas no cross reaction was seen in animals infected with a single-dose. That can be related to a booster effect on the immune system produced by the repeated infection dose, so that a stronger and non-specific immune reaction also against *Dicrocoelium* antigens could explain this cross reaction.

By SDS PAGE (Poliacrylamide Gel Electrophoresis) at least four bands in the So. antigen solution and three bands in the E/S antigen solution were detected, characterised by molecular weights of 58, 43, 24 and 15 kDa (So. antigen), and 43, 24, 15 kDa (E/S antigen). This is consistent with the results of WEDRYCHOWICZ *et al.* (1995) who also studied the immune response by bile antibodies to *D. dendriticum* somatic antigen separated by SDS PAGE. FAGBEMI and OBARISIAGBON (1991) separated the crude extract of *D. hospes* into seven peaks, but using column chromatography.

In blotting acrylamide gels on nitrocellulose membranes, some technical difficulties were encountered in obtaining a clear band pattern on nitrocelluose sheets. Some background effect was present, and many trials were attempted to limit it. It has to be borne in mind that the antigen solution was prepared by whole worm culture, where many proteic compounds are present (WEDRYCHOWICZ *et al.*, 1995) and protease factors could influence a sharp band definition negatively.

Positive sera from experimentally infected lambs reacted with *D. dendriticum* antigen fractions on nitrocellulose membrane. The results obtained showed that the immune reaction took place in the molecular weight range of 40-100 kDa, in accordance with recent studies about *Dicrocoelium* antigenic proteins (REVILLA-NUIN *et al.*, 2005), in which bands ranged from 26 up to 205 kDa.

In the present study a diffuse band was detected between 50 and 100 kDa, whereas a more defined band could be distinguished at 43 kDa, as shown in the acrylamide gel sheets stained with Coomassie blue. Low molecular weight bands of 24 and 15 kDa were present in gel electrophoresis, did not show any antigenic activity, and were absent in the immunoblot assay. At heavier molecular weight range (100-150 kDa) there was also a diffuse reaction, more evident in the somatic extracts immuno-precipitates. This result is in accordance with other authors who detected a specific parasite protein with an approximate molecular weight of 130 kDa and strong immunoreactivity against ovine sera experimentally infected with *D. dendriticum* (REVILLA-NUIN *et al.*, 2005).

Also in the study by FAGBEMI and OBARISIAGBON (1991), the strongest immune reaction to semi-purified antigen of *D. hospes* was observed at heavy molecular weight (wide peak at more than 69 kDa) whereas lower values were related to proteic fractions of light molecular weight (13.7 kDa).

The diffuse band range between 50 and 100 kDa was more evident using the So. antigen. This band range was curiously not present in stained poliacrylamide gels. Probably positive sera could react with fractions of antigens that the Coomassie staining procedure could not show. This is in accordance with other authors (TSANG *et al.*, 1983), who reported the high sensitivity of immunoblot method.

Regarding survey about time-course analysis of antibody response by immunoblot in experimentally infected lambs with *D. dendriticum*, scant literature is available.

In contrast to the results obtained by the ELISA test, in which positive sera were detected from 30 days, using immunoblot, a band pattern appeared only at 60 days post infection. In contrast, GUOBADIA and FAGBEMI (1995) found out that the *F. gigantica* infected sheep sera recognised four polypeptides in the range of 43–75 kDa as early as two weeks after infection. They also detected that an 87 kDA band was lost two weeks after treatment, and thus could be a good marker for treatment efficacy. Despite ELISA methods that are unable to quantify the success of treatment, immunoblotting could be a control method to prove efficacy of chemotherapy. This aspect could also be tested with *D. dendriticum* infections in further trials.

The band pattern was the same both using E/S and So. antigen; in the latter the pattern was more marked, probably due to the higher concentration of antigen.

A comparative immunoblot of *D. dendriticum* E/S and So. antigen with sera of sheep infected with others trematodes and nematodes was performed**.**

Seven sera of *F. hepatica*–infected sheep were tested by immunoblot with *Dicrocoelium* antigens. In the present study a cross reaction was detected at 43 kDa range in three samples, but reactions at molecular weight range of 50-100 kDa were present only in one sample, that, also, showed a high anti-*Dicrocoelium* titre also in the ELISA test. The band pattern at 50-100 kDa probably could be referred to the *D. dendriticum* specific band range, although composed of many proteic compounds which provoked the diffused pattern.

By blotting E/S extracts of *F. hepatica* QURESHI (1995) identified only one specific proteic band at 15 kDa, at a much lower molecular mass range than the present trials showed with *D. dendriticum.* Also in the survey by GUOBADIA and FAGBEMI (1995) comparative immunoblotting with sheep anti-*Paramphistomum,* anti-*Dicrocoelium* and anti-*Fasciola* sera revealed that the 17 kDa, 21 kDa, 57 kDa and 69 kDa proteins are specific for fasciolosis and

they are good antigens for early and specific immunodiagnosis of *F. gigantica* infection in sheep.

Anyway, in the present study cross reactions between anti-*Dicrocoelium* and anti-*Fasciola* positive sera were present, and the antigenic behaviour of protein fractions was not highly specific. As reported by FAGBEMI and OBARISIAGBON (1991) there is evidence that one major problem in the immunodiagnosis of helminth infections is that of the cross-reactivity of the antigens, and it is often assumed that this cross-reactivity is peculiar to crude antigens and would be removed by purification. It seems, however, that even highly sensitive methods such as gel chromatography may not achieve the degree of purification needed to separate species-specific antigens from the crude extracts. Currently, however, the technique of immunoblotting, if properly implemented, provides the most powerful means of discriminating between antibody responses that are species-specific and those that are cross-reactive (FAGBEMI and OBARISIAGBON, 1991).

Sera from sheep experimentally infected with L_3 of *N. battus, T. colubriformis* were tested in immunoessay with *D. dendriticum* antigens separated by electrophoresis. The samples were negative, and no immunoprecipitates were visible. Such a result was expected because these nematodes are zoologically unrelated to *D. dendriticum.* Such a result is encouraging, because, in the present study, *Dicrocoelium* antigens cross-reacted only with field sera of *Fasciola*-infected adult sheep, that cannot guarantee specific reactions. In order to validate properly the test specificity, lambs monoinfected with different trematodes are needed.

Although there are many publications on immunoelectrophoretical studies of antigenic products regarding common liver flukes (*Fasciola* spp.), in the case of *Dicrocoelium* spp. there is little reported, making comparisons of results difficult. Further immunoelectrophoretical trials with purified antigen are needed, in order to define the band spectrum of *D. dicrocoelium* antigenic proteins.

Although several investigations have been conducted on the prevalence of dicroceliosis in sheep using coprological analysis (FERRE *et al.*, 1994; MANGA-GONZÁLEZ *et al.*, 1991; SCALA *et al.*, 1991; JITHENDRAN and BHAT, 1996), reports of about large scale sero-epidemiological studies on dicrocoeliosis are very rare (BALDELLI *et al.*, 1981; SANCHEZ-ANDRADE *et al.*, 2003). As far as it is known, the first seroepidemiological survey of ovine dicrocoeliosis in province of Trento is reported in the present study.

The mean seroprevalence of sheep dicrocoeliosis in province of Trento observed in the present study is very high (90%), as in other epidemiological studies on dicrocoeliosis in Italy conducted in areas such as Umbria and Sardinia (AMBROSI *et al.*, 1980, SCALA *et al.*, 1991), with coprological analysis. However, since the seropositivity to dicrocoeliosis is maintained for a long period (GONZALEZ-LANZA *et al*., 2000), positive reactions can also indicate previous exposure to the parasite without current infection, as occurs also in fasciolosis (PAZ *et al*., 1998; SANCHEZ-ANDRADE *et al*., 2000, 2001). In fact, serum antibodies may be present if the animal has previously been infected, regardless of current parasitological status. Regarding this, it must be kept in mind that some of the sheep flocks examined were transhumant and during the winter they are brought down from alpine pastures along the Piave and Brenta valley, where they may be also infected by *D. dendriticum*. ELISA methods that measure antibody levels, as in our case, are unable to quantify the success of chemotherapy (LECLIPTEUX *et al*., 1998).

In this study, seroprevalence rates registered with somatic antigens were higher than those using E/S extracts, which could confirm due to the lower specificity of the former protein extract (GONZALEZ LANZA *et al*., 2000).

The highest sero-prevalence rates (95-100%) have been registered in flocks from municipalities of Telve, Torcegno, Frassilongo, Castelnuovo and Cavalese, areas that are relatively close to each other, between the two valleys of Valsugana and Val di Fiemme, separated by Lagorai mountain chain. The altitude of this area varies between 750 and 2000 m a.s.l.

The lowest seroprevalence was registered in the flock from district of Arco (E/S: 68%; So: 52%), at the northern point of Garda lake, the municipality at the lowest altitude of the study area (250 m a.s.l.).

It could be interesting to further study the seroprevalence of flocks and ecological characteristics in these two areas in order to investigate relations between seroprevalence rates, type of habitat for the intermediate hosts and differences in sheep flock management in comparison to other areas of Trentino Province.

Anyway, since dicrocoeliosis is a long-term infection that may cause chronic disease with loss of production (SCALA *et al.*, 1991), the results of the present study should be evaluated and compared with further studies, in order to define better the epidemiological

situation for planning the health management of grazing flocks in this area and in general in the mountain pastures.

Therefore the present serological survey has not to be interpreted as a mere diagnostic investigation. In fact it must be reminded that the ELISA test used is experimental and still needs improvement in accuracy, precision and reliability. Nevertheless the results have an epidemiological meaning: detecting sero-prevalence rates and distribution of *D. dendriticum* infection may be helpful in drawing epidemiological maps, and if data about the distribution status of intermediate hosts are available, it can lead to the definition of risk factors in different pastures.