

**COMPARISON OF TWO PARASITOLOGICAL TECHNIQUES FOR THE
DIAGNOSIS OF INTESTINAL PARASITES IN A POPULATION OF IBERIAN IBEX
(*Capra pyrenaica* SCHINZ, 1838) IN THE SIERRA DE GUADARRAMA
NATIONAL PARK (SPAIN)**

*(Comparação de duas técnicas parasitológicas para o diagnóstico de parasitas
intestinais numa população de cabra-montês (*Capra pyrenaica* Schinz, 1838) no
Parque Nacional de Sierra de Guadarrama – Espanha)*

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ABSTRACT: Twenty samples of Iberian ibex collected in October 2014 in Sierra de Guadarrama National Park (Spain) were analyzed to evaluate detectability of intestinal parasites by two concentration techniques: Ritchie (1948) and Anécimo *et al.* (2012) and also to analyze and compare the parasite catalogue of each technique. There has been a decrease in the diversity of species found and an increase in prevalence data with respect to previous studies carried out in the same area, possibly due to the increase in Iberian ibex density in the area. A nematode species (*Nematodirus filicollis*) that has not previously been found in the population under study and which typically parasitizes cattle, has also been identified. This suggests an exchange of parasites between wild ungulates population and domestic livestock in this area. There were no significant differences between the results obtained by the two techniques used so that, the improvement in the working conditions of the analysts that involves the use of the Anécimo technique by not using hazardous solvents to health, can serve as a guideline to change the protocol of action.

Keywords: Detectability; technique concentration stool; parasites.

RESUMO: Vinte amostras de cabra de montanha analisada neste artigo, foi recolhida em outubro de 2014, no Parque Nacional da Serra de Guadarrama na Espanha. A análise visou primeiro, avaliar a detectabilidade de parasitas intestinais com recurso duas técnicas de concentração, Ritchie (1948) e Anécimo *et al.* (2012), segundo, comparar o catálogo de parasitas para cada técnica. Os resultados da nossa análise mostram, que, em relação aos estudos anteriores realizados na mesma área: houve diminuição da diversidade de espécies, um aumento de dados de prevalência devido ao aumento da densidade de ibex na área, identificamos uma nova espécie de nemátodo (*Nematodirus filicollis*) cujos parasitas são tipicamente bovinos. Este último resultado, sugere que existe uma troca de parasitas entre a população de ungulados selvagens e o gado doméstico da área. Quanto a utilização de duas técnicas de concentração, os resultados não mostram diferenças significativas entre os obtidos tanto pela técnica de Ritchie (1948) como pela técnica de Anécimo *et al.* (2012). Por causa disso, defendemos que, para a

melhoria de condições de trabalho de técnicos implica a utilização da metodologia proposta por Anécimo *et al.* (2012), por não utilizar solventes perigosos à saúde. Esta proposta pode servir de guia para alteração do protocolo de ação.

Palavras-chave: Detetabilidade; Técnica de concentração fezes; Parasitas.

INTRODUCTION

The study of species that parasitize wildlife has greatly advanced in the last decade, progressively increasing the interest that parasites play in biodiversity and ecosystem dynamics (Hatcher and Dunn, 2011). The knowledge of the negative effect of parasites on livestock production, wildlife conservation and human health has increased their interest and the number of studies. (Barasona, 2015)

In the case of ruminants, abundance of gastrointestinal parasites can have great importance in their population dynamics (Díez-Baños and Hidalgo-Argüello, 2006); parasites can decrease reproductive success, either through a direct effect on fertility (Body *et al.*, 2011) or through a negative impact on growth and body condition (Joly and Messier, 2005). This effect may be the result of subtle interactions between the host, the parasites and the environment and therefore, may be subject to factors such as the availability of food, infections by other pathogens, etc. (Smith *et al.*, 2005).

Several studies indicate the influence of parasites on the reproductive success of different wild mammals without the need to produce apparent pathogenicity (Marzal *et al.*, 2005; Tompkins *et al.*, 2011); they can also directly or indirectly influence the dynamics of their populations affecting their vulnerability, predation or competition for trophic resources (Hernández *et al.*, 2001; Wisnivesky, 2003; Hudson *et al.*, 2006; Audesirk *et al.*, 2008; Tompkins *et al.*, 2011). These effects will be conditioned by the general condition of the animal (Wisnivesky, 2003), age, type of feeding, way of life, climate and seasonality (Quiroz, 2005). Parasitic infections in Iberian ibex are frequent, serious and well documented, with about a hundred parasites described. (Refoyo *et al.*, 2016).

The main problem when performing a parasitological study is the manipulation of the host, especially when dealing with large mammals such as ungulates. Although the coprological analysis has become a very effective technique to obtain information on parasites that affect wildlife without resorting to invasive techniques (Balmori *et al.*, 2000; Alasaad *et al.*, 2008; Beltrán-Saavedra *et al.*, 2009), it limits its study to the forms that live in the intestine or that eliminate their infectious forms through it (Cordero del Campillo and Rojo, 2000). In addition, the study of the coprological samples allows in the obtaining of complementary data of the studied population such as taxonomically identify the diets, measure hormones to study the relationship with the reproductive behaviour or establish the different trophic interrelationships in an ecosystem (Beltrán-Saavedra *et al.*, 2009).

To face a parasitic coprological analysis there is a series of techniques that allows the identification of different forms of dispersion of parasitic species such as trophozoites, cysts and oocysts in the case of protozoa and eggs and larvae in the case of helminths (Cordero del Campillo and Rojo, 2000). In order to concentrate the different forms that can appear in a sample, it is necessary to use different enrichment techniques such as sedimentation, flotation or combining both, to increase the sensitivity of the observation when the number of parasitic forms is low and there is absence in the direct examination of the sample (Salvatella and Eirale, 1996). Among the most used techniques of sedimentation is the Ritchie technique and among the flotation techniques are Willis (saturated sodium chloride solution) or Faust (zinc sulfate solution) (Salvatella and Eirale, 1996; Cordero del Campillo and Rojo, 2000).

There are several works that use these and other techniques of concentration in different groups of animals. In ungulates, the Ritchie technique or any of its modifications (Teleman technique, modified Ritchie, miniParasep, etc.) and the Willis technique, obtain good results (Alasaad *et al.*, 2008; Refoyo, 2012). Different studies have also been carried out comparing the detectability and effectiveness of the different techniques, both sedimentation versus flotation and between the different sedimentation techniques (McNabb *et al.*, 1985; Beltrán *et al.*, 2003; Del Coco *et al.*, 2008; Tenorio-Abreu *et al.*, 2013; Barba, 2015; Barbosa *et al.*, 2016).

Nowadays, modifications of coprological techniques are increasing in order to avoid the use of chemical solvents and replacing them with neutral solvents and, therefore, reducing the risk of exposure of health professionals and avoiding the generation of potentially polluting residues. In those techniques, the ether is replaced by different solvents, the formaldehyde by distilled water or ethyl alcohol in different concentrations (Del Coco *et al.*, 2008; Anécimo *et al.*, 2012; Tenorio-Abreu *et al.*, 2013). All techniques show very good results in human samples but have not been used in herbivorous animals, except the MiniParasep commercial kit which uses formaldehyde and neutral soap in a capsule with a filter with a pore size of 425µm and which, in the case of the Iberian ibex, has not given satisfactory results in the study of parasitic load compared to the Ritchie technique but adequate in diversity studies (Barba, 2015).

In this work, the Ritchie technique has been compared with the modification proposed by Anécimo *et al.* (2012) that replaces formaldehyde by hot distilled water and ether by neutral soap in a population of Iberian ibex in a protected natural area.

Our aim is to compare the detectability and efficacy to study the parasitic load between the Ritchie technique (Ritchie, 1948) and the modified technique proposed by Anécimo *et al.* (2012) in order to assess the risk of exposure to chemical solvents by analysts in the event that no significant differences were observed between the two techniques in a population of Iberian ibex of the Sierra de Guadarrama National Park (Spain).

MATERIALS AND METHODS

In October 2014, samples of fresh faeces were collected from 20 adult individuals of a population of Iberian ibex from the Sierra de Guadarrama National Park (Spain) at an altitude of 2000 meters (Figure 1). This national park has an area of 33,960 ha and it is located in the centre of the Iberian Peninsula, in the eastern part of the Central System mountains.

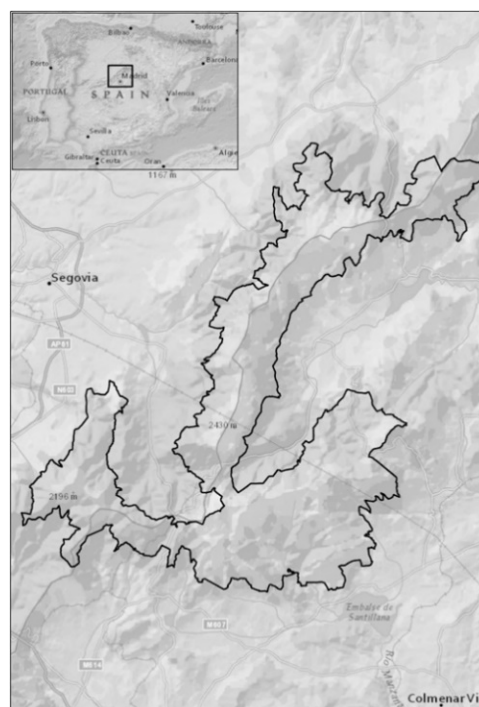


Figure 1. Study area.

Faeces samples were collected directly from the soil to avoid the use of

invasive techniques that cause stress in the population. Sampling was done at dawn by locating groups of mountain goats in the study area. Once the groups were located, we wait for them to move to collect fresh faeces from the animals ensuring a minimum distance between faeces to be able to individualize the samples, as indicated by Acevedo *et al.* (2005) and Acevedo *et al.* (2011). Therefore, only one sample was selected from each group (20 groups), thus avoiding possible pseudoreplications.

The samples were collected in independent plastic bags and properly labeled with the sample number. They were transported fresh and in the laboratory were sieved using distilled water and kept in a refrigerator at 4°C for further processing, according to Carvalho *et al.* (2012). They were kept at room temperature for one week in a solution of sodium acetate buffer pH 5 to allow the oocysts sporulation (Ayres and Mara, 1997).

The samples were sieved using 1mm mesh of light in order to separate the faecal remains from other possible elements (plants, minerals, etc.) that could have been mixed with the faeces (Fernández de Mera, 2007). Next, part of the sample was centrifuged with saline solution at 2500 rpm for one minute to remove the sodium acetate buffer pH 5, discarding the supernatant and the processed solution was poured into tubes containing 10 mL of saline solution and the centrifugation was repeated for one minute at 2000 rpm until obtaining translucent supernatants.

Thus, from each of the obtained samples, 3 g were processed following the Ritchie's concentration technique by sedimentation (Salvatella and Eirale, 1996) and other 3 g following the protocol of the Ritchie modified technique by Anécimo *et al.* (2012). After processing all samples by each technique, 100µL was taken with a 1mL

pipette to make a microscopic preparation adding a drop of iodine solution and observing it under an optical microscope. The search for parasitic forms was carried out using the objective of 10x and 40x and was measured (major axis and minor axis) and also photographed using a micrometric eyepiece with the objective of 40x. Protozoa and helminths are identified by morphometric analysis according to Kaufmann (1996), Cordero del Campillo and Rojo (2000), Quiroz (2005) and Refoyo (2012). But this type of analysis is problematic due to the existence of cryptic and polymorphic species (Indre *et al.*, 2010; Yang *et al.*, 2014). However, both larvae and adults of helminths have taxonomic characteristics that allow a more precise determination (Van Wyk *et al.*, 2003; Van Wyk and Mayhew, 2013). Nevertheless, morphometric data are compared with those obtained by other authors in the study area for the same host (Refoyo, 2012; Barba, 2015; Refoyo *et al.*, 2016).

A statistical analysis was carried out to compare the detectability of the two techniques used considering values of $P < 0,05$ as significant. In this sense, a nonparametric test to determine the significance of the two techniques was used, the Mann-Whitney test, as well as the Kruskal-Wallis test to confirm the significance of the variables used in the two techniques. Statistical analyses were performed using STATISTICA version 7.0 (StatSoft Inc., Tulsa, Oklahoma, USA).

RESULTS

Coccidia were detected in 17 of the 20 analyzed samples (85%) while nematodes were detected in 16 of the 20 samples (80%) (Table 1). However, prevalence slightly varies when we observe independently each technique. In the Ritchie technique, coccidian

appears in 75% of the samples and in the Anécimo technique increases up to 80%. Considering the group of nematodes, in the Ritchie technique prevalence was 60% compared to 65% in the Anécimo technique (Table 1). For

all that, the two sedimentation techniques can be used to search and identify coccidia and nematodes although the Anécimo technique seems to be more effective for both groups.

Table 1. Prevalence data for each of the two techniques used for the two groups of parasites observed by sample.

SAMPLE	Coccidia		Nematodes	
	R ¹	A ²	R ¹	A ²
1	0	1	0	1
2	1	1	1	1
3	1	1	0	1
4	1	1	1	0
5	1	0	1	1
6	0	0	0	0
7	1	1	1	0
8	1	1	0	1
9	0	0	0	0
10	0	0	0	0
11	1	1	1	1
12	1	1	1	1
13	0	1	1	1
14	1	1	1	1
15	1	1	1	1
16	1	1	1	0
17	1	1	0	1
18	1	1	1	1
19	1	1	1	1
20	1	1	0	0
Total	15	16	12	13
PREVALENCE (%)	75	80	60	65

¹ Ritchie technique ² Anécimo technique

Having into account these values, we see that the most prevalent coccidium species in the two techniques has been *Eimeria christensen* with 55% in the Ritchie and 60% in the Anécimo, followed by the *E. arloingi-cylindrica* complexes and *E. caprina-caprovina* (40% in both techniques for the first case and 35% and 55% for Ritchie and Anécimo techniques for the second, respectively) (Table 2). Due to its scarce morphological differences and to reduce the risk of error in the identification of

coccidia, we have decided to group the species into complexes because of the great difficulty of identifying each species in an individualized way.

If we now analyze the obtained data by each technique separately, we observe that there are hardly any differences except for *E. ninakohlyakimovae* which has only been detected by the Anécimo technique and with a rather lower prevalence compared with the rest of the species. This may be since the detectability is improved with

the Anécimo technique because the preparations are clearer because of the high dispersion of the organic matter

fragments thanks to the use of the detergent.

Table 2. Prevalence data for each of the two techniques used by sample.

SAMPLE	Coccidia								Nematoda							
	Ech ³		Ear-cy ⁴		Eca-co ⁵		Eni ⁶		Nsp ⁷		Nfi ⁸		Mca ⁹		Dvi ¹⁰	
	R ¹	A ²	R ¹	A ²	R ¹	A ²	R ¹	A ²	R ¹	A ²	R ¹	A ²	R ¹	A ²	R ¹	A ²
1		1										1				
2	1	1								1			1			
3	1		1	1	1	1						1		1		
4	1		1	1		1		1	1				1			
5			1		1				1	1	1	1	1	1		
6																
7	1	1	1			1									1	
8	1	1				1				1				1		
9																
10																
11				1	1			1	1		1		1	1		
12			1		1	1			1	1			1	1		
13		1								1		1	1			
14	1	1	1	1	1	1		1	1		1		1	1		
15	1	1		1		1		1					1			1
16	1	1		1	1	1			1				1			
17	1	1	1		1	1			1					1		
18	1	1		1		1			1				1	1		
19	1	1		1		1		1	1	1		1	1	1		
20		1	1													
Total	11	12	8	8	7	11	0	5	7	8	3	5	11	9	1	1
PREVALENCE (%)	55	60	40	40	35	55	0	25	35	40	15	25	55	45	5	5

¹Ritchie technique; ²Anécimo technique; ³*Eimeria christensenii*; ⁴*E. arloingi-cylindrica* complex; ⁵*E. caprina-caprovina* complex; ⁶*E. ninakohlyakimovae*; ⁷*Nematodirus spathiger*; ⁸*N. filicollis*; ⁹*Muellerius capillaris*; ¹⁰*Dictyocaulus viviparus*.

In addition, the oocysts of this species are small, so it is easy for them to be concealed by the remains of organic matter and, therefore, it is much more difficult to detect them using the Ritchie technique. When analyzing whether there are differences in the detectability of both techniques with Mann-Whitney test for: number of parasites detected (U(1-120)= 6947.00; P=0.638), number of species (U(1-120)= 7076.50; P=0.8013) and by groups found for both coccidia (U(1-120)= 6798.50; P=0.4553) and nematodes (U(1-120)= 6852.00; P=0.5175), in

general and by species (Coccidian U(1-120)= 6866.50; P=0.5351) (Nematodes U(1-120)= 6868.00; P=0.5369), no significant differences were detected (P<0,05) between the two techniques for none of the variables analyzed.

Considering each of the variables analyzed independently (number of parasites and diversity of parasites both globally and by groups), no significant differences were detected (Box-Plot) for each technique (Figures 2, 3 and 4).

In relation to the box plots exposed above, for all the analyzed variables (global parasites, coccidia and

nematodes), it seems that both techniques (Ritchie and Anécimo) have the same sensitivity in relation to the reliability in detecting intestinal parasites in the samples of Iberian ibex faeces observed in this work.

When comparing the two techniques with respect to the presence of artifacts (remains of organic matter) in the preparations studied under the

microscope, it has been observed that samples processed by the Ritchie technique had a higher concentration of particles or sediments than preparations made with the treatment of Anécimo technique which at some point, interferes with visualization as can be seen in the below selected samples (Figure 5, 6 and 7).

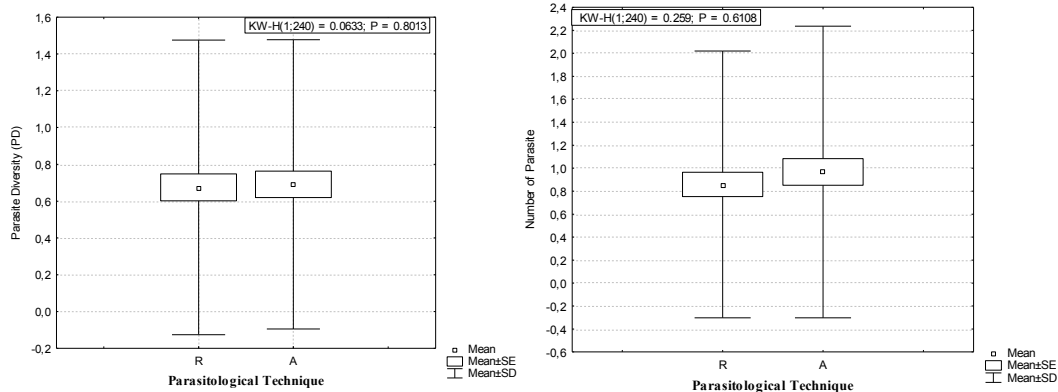


Figure 2. Diversity of parasites by sample. Left number of species and right number of individuals. R: Ritchie technique; A: Anécimo technique. KW-H: Kruskal-Wallis test.

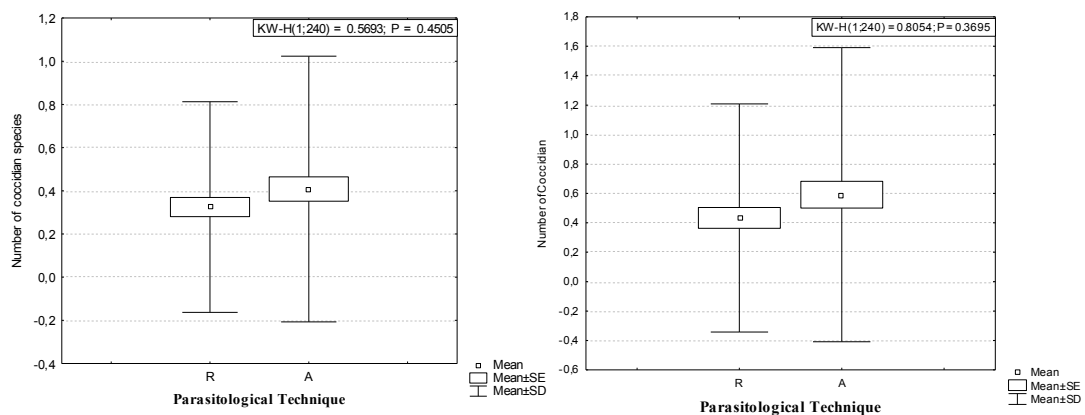


Figure 3. Diversity of number of Coccidia by sample. Left number of species and right number of individuals. R: Ritchie technique; A: Anécimo technique. KW-H: Kruskal-Wallis test.

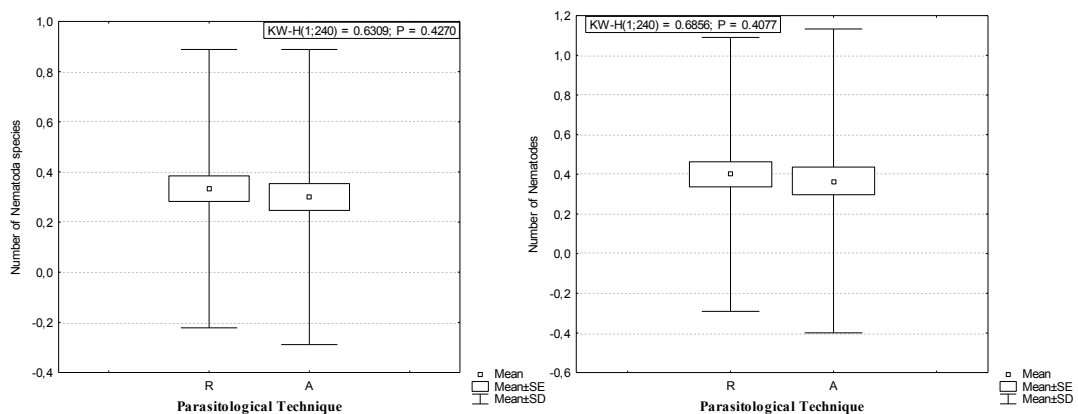


Figure 4. Diversity of nematodes by sample. Left number of species and right number of individuals. R: Ritchie technique; A: Anécimo technique. KW-H: Kruskal-Wallis test.

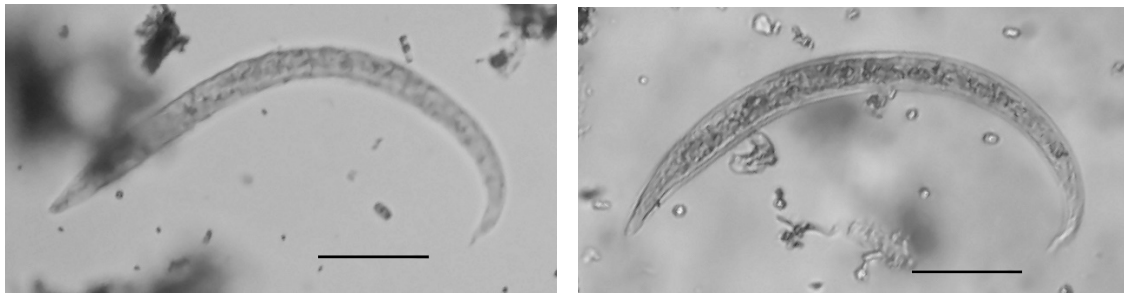


Figure 5. Larvae of *Muellerius capillaris* observed under the microscope after being treated with the Ritchie (left) and Anécimo (right) techniques. Scale bar: 50 μ m.

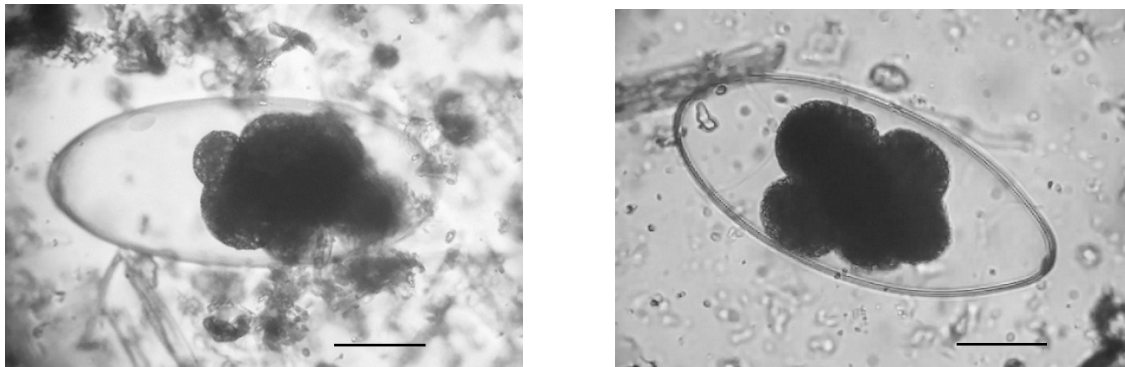


Figure 6. *Nematodirus spathiger* eggs observed under a microscope after being treated with the Ritchie (left) and Anécimo (right) techniques. Scale bar: 50 μ m.

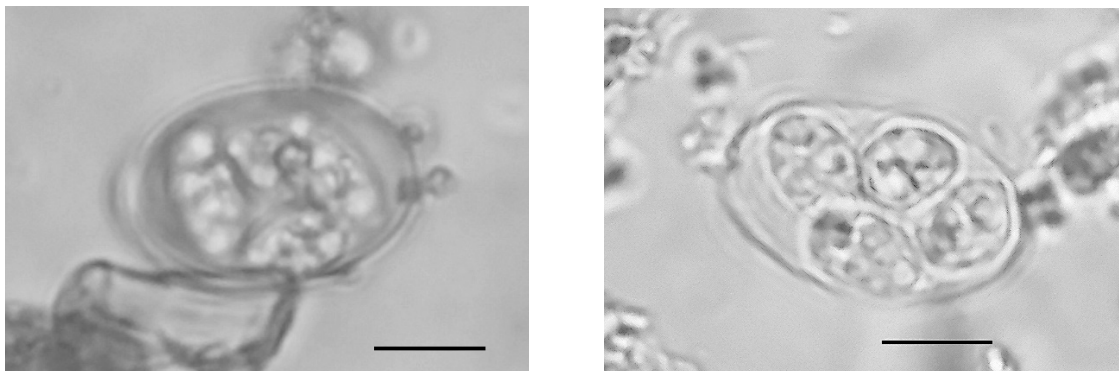


Figure 7. Oocysts of the *Eimeria caprina-caprovina* complex observed under the microscope after being treated with the Ritchie (left) and Anécimo (right) techniques. Scale bar: 10 μ m.

DISCUSSION

Coprological studies to identify the parasitosis that affect animals provide reliable data of both biological and sanitary interest. This methodology allows the observation of the first stages of the development of parasites, eggs, oocysts and larvae of the digestive and respiratory system (Balmori *et al.*, 2000) and the use of concentration techniques is essential to increase the detectability of organisms, especially in samples

where abundance is low (Saez *et al.*, 2012).

Regarding coccidia, the Iberian ibex is parasitized fundamentally with the genus *Eimeria*, its prevalence in the studied populations in Spain oscillates between 43% in Salamanca (Ramajo *et al.*, 2007) and 74% in Andalucía (Pérez Jiménez, 2005), south of Spain. In previous studies carried out in the study area, prevalence of 83.3% has been obtained (Barba, 2015). Our results agree with these data since the

prevalence of this group of protozoa oscillate between 75% and 80% according to the method used for the analysis.

The species of coccidia located in this study match with those cited for wild goat by Alados and Escós (2012), although we have not found *Eimeria hirci*, a typical goat species that has not been previously mentioned in the study area (Refoyo, 2012; Barba, 2015). This may be due to the low prevalence reported in this host (Ruiz *et al.*, 2006). However, we do find *Eimeria cylindrica*, a typical species of cattle (Cordero del Campillo and Rojo, 2000), abundant in the area and which has also appeared in the previous works (Refoyo, 2012; Barba, 2015).

Species with the highest prevalence data in this study has been *Eimeria christensenii* with values ranging between 55% and 60%, very high values when compared with those obtained by Refoyo (2012) which place it at 25% and those of Barba (2015) that oscillate between 10% and 18%, according to the technique used for their detection. In our study, prevalence data for nematodes ranging from 60% to 65%, very high values when compared with those obtained by Barba (2015) with 34.4% but somewhat low for those found by Refoyo (2012) that reached 80%. However, we have found a very low diversity since only 4 species have appeared unlike these authors detect 11 and 13 species, respectively.

The difference in results may be due to differences in the methodology followed (Refoyo, 2012; Barba, 2015). Refoyo (2012) conducts an annual follow-up study of the population taking samples in spring, summer and autumn, giving an average prevalence figure throughout the year. Barba (2015) carries out a sampling campaign in spring while we have done this work in the autumn season. This difference in the sampling campaigns affects the

results obtained since the behaviour of the Iberian ibex population varies considerably throughout the year with small groups of animals being observed in the spring compared to the large herds observed in the fall, which could increase the risk of infection of animals.

As for *Nematodirus spathiger*, Barba (2015) obtains 1% prevalence while Refoyo (2012) does it in 15% of the samples. In this study we obtain higher data, reaching 35-40%. In addition, we have found *Nematodirus filicollis* with a prevalence ranging between 15-25% that had not been previously detected in the population under study; this species is common in cattle and other ruminants, so it is not uncommon to expect its occurrence in the Iberian ibex population with which it has a close relationship.

High prevalence data may be due to the strong increase of the ibex population in the study area which has gone from 6.57 ind./km² in 1992 to 44.82 ind./km² in the 2014 sampling campaign (Refoyo *et al.*, 2014). The increase in the density of ungulates in certain areas results in an increase in the population of hosts available for parasites and the possible appearance of highly parasitized animals. These, because they have a lower capacity to compete for resources, move to areas where food is scarce causing the alteration of the host habits and even their disappearance which leads the parasite to look for new hosts to which it is not adapted and that, as a consequence, the risk of contagion and the appearance of epizootics increase (Díez-Baños and Hidalgo-Argüello, 2006; Granados *et al.*, 2007).

Regarding the comparison made between different sedimentation methodologies in the study of coprological samples, it should be noted that no significant differences were found for any of the variables studied. These data coincide with those obtained

by Anécimo *et al.* (2012) for human faeces in which they did not find relevant differences of detection neither for different groups of protozoa nor for helminths.

Ritchie technique is a methodology routinely used in laboratories because it isolates a wide variety of parasitic forms of faeces, both in fresh and preserved samples and in addition, it is a very sensitive technique to detect mild infections (Funk *et al.*, 2013). However, the use of formaldehyde and ether solvents makes the manipulation of the samples dangerous for operators and attempts to modify the original methodology to reduce or prevent risks in the laboratory.

Under this premise, Barba (2015) compared two techniques used in human coprology with the Ritchie technique to adapt them to routine work with ruminant samples. Their most outstanding results indicate that the use of a commercial kit (Mini Parasep SF®) which does not need solvents and uses two filters of different mesh, shows results very similar to Ritchie technique when it comes to studying the parasitic diversity and therefore, this should be the technique of choice for conducting this type of study since it offers minimal manipulation, shorter processing time, lower cost and decreases the analyst risk of work. These results also coincide with those obtained by Tenorio-Abreu *et al.* (2013) for human faeces. However, Saez *et al.* (2011) warns of the use of this kit can cause false negatives since parasitic forms of smaller size can be lost in the manipulation of the sample or stay in the filters that collapse.

The technique used in this work offers consistent results. It is even more economical and as not uses filters, the sample collapse is avoided and for that, probability of detection increases. Samples analyzed with the Anécimo technique show a greater clarity what

facilitates parasitic forms to be better detected, something that does not happen with the Ritchie technique in which a greater amount of dispersed organic matter is observed, as pointed out by Barba (2015) in their comparison between techniques.

CONCLUSIONS

As the main conclusions of this study, we can indicate that although there has not been a sanitary deterioration of the Iberian ibex population under study, a considerable increase has been detected in the prevalence data of the species found with respect to previous works. However, we have found a decrease in the diversity of species that affect the population although a new species of nematode, typical of cattle, has been identified. These facts seem to be related the significant increase in the density of ibex in the area and can support the hypothesis, sustained by other authors, of the exchange of parasites between the populations of wild ungulates and domestic livestock.

On the other hand, the results obtained in this work seem to indicate that the Anécimo technique can be used with guarantees for the study of intestinal parasites in ungulates, since no significant differences have been observed with the technique used as a reference in this type of studies. The improvement in the working conditions of the analysts by not using dangerous solvents to health can be a start to change the protocol of action. However, new studies with other ungulates and a greater number of samples will be necessary for this to be chosen as a reference technique.

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