UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE VTERINARIA

Departamento de Sanidad Animal

TESIS DOCTORAL

Efecto antihelmíntico y nutritivo del brezo en ganado caprino

Antihelmintic and nutritional effect of heather in goats

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Javier Moreno Gonzalo

Directores

Ignacio Ferre Pérez, Koldo Osoro Otaduy

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Las nematodosis gastrointestinales de los pequeños rumiantes son las enfermedades parasitarias más frecuentes e importantes en los sistemas de explotación extensivos y semiextensivos. El método de control más utilizado frente a estas parasitosis es la administración repetida de fármacos antihelmínticos en las épocas de mayor riesgo.

Sin embargo, la aparición de cepas de nematodos resistentes a los fármacos más utilizados y la demanda de los consumidores de productos naturales y libres de cualquier residuo ha estimulado la búsqueda de nuevas estrategias de control.

La presente Tesis Doctoral ha estudiado el efecto antihelmíntico y nutritivo del brezo en el ganado caprino. Se ha investigado el efecto de la suplementación con brezo en ganado caprino en pastoreo e infectado por nematodos gastrointestinales de forma natural, observándose una mejora en su ganancia de peso y condición corporal y una reducción de la mortalidad y de la excreción de huevos de nematodos gastrointestinales en las heces. Mediante infecciones experimentales con *Trichostrongylus colubriformis y Teladorsagia circumcincta* se demostró que el consumo de brezo reduce la implantación de larvas infectantes y la excreción fecal de huevos, asociándose este último hecho a una disminución del desarrollo y la fecundidad de los vermes adultos. Por último, mediante pruebas *in vitro* se demostró el efecto antihelmíntico de extractos de diferentes especies de brezo sobre varios estadios de desarrollo de *Trichostrongylus colubriformis*, *Teladorsagia circumcincta* y *Haemonchus contortus*. Se observó disminución en la eclosión de huevos, reducción de la tasa de desenvainamiento de las larvas infectantes y descenso de la supervivencia de los vermes adultos.

Mediante las infecciones experimentales y los experimentos *in vitro* se corroboraron los resultados obtenidos en condiciones naturales, demostrándose que el consumo de brezo por las cabras en pastoreo debería ser contemplado como un método alternativo o complementario en el control de las nematodosis gastrointestinales de estos animales.

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J. Moreno Gonzalo

2013

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Doctoral Thesis

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Memoria presentada por Don Javier Moreno Gonzalo para optar al grado de Doctor por la Universidad Complutense de Madrid

Madrid, 12 de junio de 2013

D. Ignacio Ferre Pérez, Doctor en Veterinaria y Profesor Titular adscrito al Departamento de Sanidad Animal de la Facultad de Veterinaria de la Universidad Complutense de Madrid y D. Koldo Osoro Otaduy, Doctor en Veterinaria, responsable del área de investigación en Sistemas de Producción Animal y Director Gerente del Servicio Regional de Investigación y Desarrollo Agroalimentario del Principado de Asturias (SERIDA)

CERTIFICAN:

Que la Tesis Doctoral titulada: "Efecto antihelmíntico y nutritivo del brezo en ganado caprino" que presenta el Licenciado en Veterinaria Don Javier Moreno Gonzalo, para optar al grado de Doctor por la Universidad Complutense de Madrid con Mención Europea, ha sido realizada en las dependencias del Departamento de Sanidad Animal de la Facultad de Veterinaria de la Universidad Complutense de Madrid y en la Finca Experimental de zonas marginales del SERIDA, localizada en la Sierra de San Isidro en Illano (Asturias) bajo su supervisión y cumple todas las condiciones exigidas para optar al grado de Doctor por la Universidad Complutense de Madrid con Mención Europea.

De acuerdo con la normativa vigente, firmamos el presente certificado, autorizando su presentación como directores de la mencionada Tesis Doctoral.

En Madrid, a doce de junio de dos mil trece.

Fdo. Dr. Ignacio Ferre Pérez

Fdo. Dr. Koldo Osoro Otaduy

A todos los que han creído que esta tesis

merecía su esfuerzo

"Mañana despertaré

y empezaré de cero

hay tantas cosas que quiero hacer

que antes me daban miedo

y aunque me pierda antes de salir

mirando el lado bueno

tengo el defecto de sonreír

sólo por no estar muerto…

...no hay más razón

que un corazón

siempre loco por vivir……"

***QUÉ NECESARIO ES EL ROCK&ROLL*, Adolfo Cabrales**

"El experimentador que no sabe lo que está

buscando no comprenderá lo que encuentra"

Claude Bernard (1813-1878) Fisiólogo francés

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"El que da nunca debe volver a acordarse; pero el que recibe nunca debe olvidar".

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La importancia de este trabajo llamado “tesis doctoral” como de todo lo demás es relativa, pero también es cierto que “fue el tiempo que pasaste con tu rosa lo que la hace tan importante” y yo he pasado bastante tiempo con ésta. En esta tesis he dejado un trozo de mi vida y sobre todo he aprendido mucho, es un paso más que he dado y un paso menos que me queda. El camino que todo tome ahora es importante, pero no definitivo, lo que nos lleva al final son nuestros pasos, no el camino, y tras esto sencillamente estoy más preparado para seguir adelante.

“Uno puede devolver un préstamo de oro, pero está en deuda de por vida con aquellos que son amables”. Por eso, la mejor manera de devolveros todo lo que habéis hecho son unas enormes y sinceras **GRACIAS**.

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Capítulo III. INFECCIONES EXPERIMENTALES

Capítulo IV. EXPERIMENTOS *In vitro*

Estructura de la tesis

Esta tesis doctoral se presenta como compendio de publicaciones. La introducción es una revisión de las últimas investigaciones sobre el control de las nematodosis gastrointestinales mediante la utilización de plantas bioactivas. Además se resumen los trabajos realizados utilizando brezo en el ganado caprino. La parte final de la introducción incluye los objetivos generales y específicos de la tesis. Cada uno de los capítulos siguientes incluye dos artículos científicos en diferente estado de publicación que se presentan tal y como se editarán en la revista científica original. En ellos se ha investigado el efecto antihelmíntico del brezo mediante diversos estudios que incluyeron cabras en pastoreo infectadas de forma natural, cabras infectadas experimentalmente y, por último, experimentos *in vitro*. Después, se ofrece una discusión general sobre los resultados obtenidos en los trabajos mencionados. A continuación, se enumeran las conclusiones que hemos podido extraer en esta tesis doctoral. El último apartado corresponde a la bibliografía. Como cada artículo incluye su propia bibliografía, en la bibliografía final solo se incluyen las referencias que no forman parte de dichos artículos.

Índice de abreviaturas/ Abbreviation index

ADF	Acid detergent fibre	Fibra ácido detergente
ADL	Acid detergent lignin	Lignina ácido detergente
ANOVA	Analysis of variance	Análisis de varianza
a.s.l.	Above sea level	Sobre el nivel del mar
BCS	Body condition score	Índice de condición corporal
BW	Body weight	Peso vivo
CT	Condensed tannins	Taninos condensados
CP	Crude Protein	Proteína cruda
DM	Dry Matter	Materia seca
DMD	Dry matter disappearance	Desaparición de la materia seca
epg	Eggs per gram	Huevos por gramo
FEC	Fecal egg count	Recuento fecal de huevos
GI	Gastrointestinal	Gastrointestinal
GIN	Gastrointestinal nematodes	Nematodos gastrointestinales
ivTSD	*In vitro* true substrate digestibility	Digestibilidad real *in vitro* del sustrato
L3	Third stage larvae	Larvas de tercer estadio
LW	Live weight	Peso vivo
NDF	Neutral detergent fibre	Fibra neutro detergente
OM	Organic Matter	Materia Orgánica
PBS	Phosphate buffered saline	Tampón fosfato salino
PEG	Polyethylene glycol	Polietilenglicol
p.i.	Post-infection	Postinfección
S.E.	Standard error	Error éstandar
S.E.M.	Standard error of the mean	Error estándar de la media
S.D.	Standard deviation	Desviación estándar
SR	Stocking rate	Carga ganadera
TP	Total phenols	Fenoles totales
TT	Total tannins	Taninos totales
VFA	Volatile fatty acid	Ácidos grasos volátiles
vs	*Versus*	*Versus*

CAPÍTULO I

Introducción y objetivos

Potential use of heather to control gastrointestinal nematodes in goats

J. Moreno-Gonzalo[a], I. Ferre[a,*], R. Celaya[b], P. Frutos[c], L.M.M. Ferreira[d], G. Hervás[c], U. García[b], L.M. Ortega-Mora[a], K. Osoro[b]

[a]Grupo SALUVET, Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Ciudad Universitaria s/n, 28040-Madrid, Spain

[b]Servicio Regional de Investigación y Desarrollo Agroalimentario (SERIDA), Área de Sistemas de Producción Animal, Consejería de Medio Rural y Pesca, Principado de Asturias, 33300-Villaviciosa, Spain

[c]Instituto de Ganadería de Montaña, CSIC-ULE, Finca Marzanas, 24346-Grulleros (León), Spain

[d]CECAV, Departamento de Zootecnia, Universidade de Trás-os-Montes e Alto Douro, PO Box 1013, 5000-911 Vila Real, Portugal

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Abstract

In the last decade, numerous studies have been carried out to evaluate the potential anthelmintic benefit of the consumption of bioactive plants in small ruminants, in order to reduce the dependence on conventional chemotherapy and supporting a sustainable control of gastrointestinal (GI) parasitism. This review summarizes the anthelmintic and nutritional effects of heather (shrub species belonging to the Ericaceae family, such as *Erica* spp. or *Calluna vulgaris*) supplementation in grazing goats naturally infected by GI nematodes. The experiments were carried out in a mountain area in north-western Spain where shrubby heather-gorse vegetation is dominant. Some plots were established, in which the vegetation had been improved by soil ploughed and dressing and sowing perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*), and removing any heather that was present. Cashmere goats reared outdoors under pasture conditions were used in the experiments. The trials compared the response to GI nematode infections, animal performance and nutrition in goats supplemented or not with heather. Interactions between heather availability and other alternative methods to control GI nematode infections based on grazing management (stocking rate) or nutrition (energy supply) as well as the potential adaptation of the rumen microbiota to the consumption of tannins, were also studied. The results suggest that (i) heather supplementation in grazing goats significantly reduces the level of GI nematode egg excretion, (ii) the faecal nematode egg count reduction could be associated with a decrease in worm fertility and/or reduction in the establishment of incoming third-stage larvae, (iii) consumption of heather is associated with an apparent greater resilience of goats to GI nematode infections, and (iv) the amount of tannins consumed by the goats supplemented with heather does not seem to be associated to anti-nutritional effects which eventually resulted in a better animal performance in the animals incorporating these shrubs in their diet. Practical application of this knowledge in temperate areas would support the management of plots integrating improved pastures with high nutritive value (ryegrass-white clover) with natural vegetation communities.

Keywords: Goat, Ericaceae, Tannin, Gastrointestinal nematode, Anthelmintic control, Animal performance

1. Promising use of plants as nutraceuticals in goats

Grazing small ruminants are commonly parasitized by gastrointestinal (GI) trichostrongyle nematodes (sub-order Trichostrongylina) worldwide. In goats at pasture, GI nematodes infection reduces the efficiency of production by decreasing voluntary feed intake, live weight gain, milk yield, and carcass quality. In addition, they have a detrimental effect on the general welfare of the infected animals (Coop and Kyriazakis, 2001; Hoste et al., 2005b). To control these parasitic infections, the repeated administration of broad-spectrum anthelmintic drugs integrated, where practical, with grazing and management strategies, is the conventional method. However, the intensive chemoprophylaxis with anthelmintic drugs has led to a widespread development of anthelmintic-resistant populations of parasitic nematodes, particularly in sheep and goats, in both northern and southern hemispheres (Kaplan, 2004). Furthermore, there is an increasing public concern over drug residues in meat and milk products, and a potential risk for environmental contamination (Waller and Thamsborg, 2004). Anthelmintics may be also unavailable in developing countries due to their high cost. For these reasons, non-chemical strategies for the control of GI nematode parasites are being investigated (Ketzis et al., 2006; Stear et al., 2007). In small ruminants, alternative approaches for control of GI nematode infections include grazing management, biological control using nematode-destroying fungi, nutritional supplementation, development of vaccines, utilization of genetic resistance, and feeding or grazing plants containing bioactive compounds. For a rationale use of these novel approaches, Stear et al. (2007) suggest that no method could be recommended excluding all others, and that the combination of methods would be the optimal strategy.

Host nutrition can directly affect GI nematodes through the ingestion of plant compounds or nutrients that may penalize parasite fitness or alter the gut (nutritional) environment in which the parasites reside (Hoste et al., 2005b, 2008). Recent *in vivo* and *in vitro* studies, showed that bioactive plants containing secondary metabolites, such as tannins, sesquiterpene lactones and flavonol glycosides, are a promising option for use in integrated nematode control in farm production systems (Brunet et al., 2008a,b; Hoste et al., 2006; Terrill et al., 2007, 2009). These plants with anthelmintic properties, known as nutraceuticals, are considered for their beneficial effects on health rather than for their direct nutritional value. The supplementation with bioactive plants in goats can enhance their ability to regulate the biology of parasite worm population (resistance) as well as their ability to withstand the negative pathophysiological effects of nematode infections (resilience) (Hoste et al., 2005b).

Condensed tannins (CT) or proanthocyanidins, polymers of flavonoid units (flavan-3-ols, flavan-3, 4 diol), have been associated to ruminant nutrition benefits by increasing post-ruminal protein availability and thus reducing the consequences of GI nematode parasitism (Waghorn, 2008). Most studies on the anthelmintic properties of CT, either in experimentally- or naturally infected sheep, show a reduction in nematode burdens, worm female fecundity and faecal egg count (FEC) when a moderate concentration of CT are consumed and could, therefore, contribute to modulate the epidemiology of these parasitic diseases (Hoste et al., 2006). Recently, the number of reports showing the anthelmintic effect of CT rich plants on goats, naturally- and experimentally infected by GI nematodes, has increased. As previously described for sheep, a reduction in egg excretion and in fecundity of the female worms have been reported in goats fed bioactive plants such as sainfoin (*Onobrychis viciifolia*, Hoste et al., 2005a; Paolini et al., 2003b, 2005b), sericea lespedeza (*Lespedeza cuneata*, Min et al., 2004, 2005; Moore et al., 2008; Shaik et al., 2006; Terrill et al., 2007, 2009), sulla (*Hedysarum coronarium*, Pomroy and Adlington, 2006), and *Viscum verrocosum* (Madibela and Jansen, 2003). These results were obtained in trials where goats were naturally- or experimentally infected by GI nematodes, but under controlled conditions. However, data from grazing goats fed on tanniferous plants and naturally infected with a mixed GI nematode burden are scarce in the literature (Kabasa et al., 2000; Min et al., 2004, 2005; Moore et al., 2008). The feeding behavioural differences between sheep (mainly grazer) and goats (mainly browser), and various physiological and metabolic adaptations of goat favouring the consumption of nutritional sources rich in potentially toxic metabolites, such as CT, indicate that goats are better adapted to exploit secondary compounds rich range vegetation (Cheeke and Palo, 1995; Hoste et al., 2005b). Furthermore, the efficacy required for novel control methods, such as CT, not only should be shown in controlled laboratory studies, but also a confirmation in farm-based trials showing a reliable economic benefit is needed (Ketzis et al., 2006).

2. Studies on heather supplementation in grazing goats

Heather-gorse communities are frequent in the botanical composition of natural vegetation in mountain communities of humid temperate areas such as northern Spain. Heather includes shrub species belonging to the Ericaceae family, such as *Erica* spp. or *Calluna vulgaris*, with a relatively high content of CT but low nutritive quality (Frutos et al., 2002). Goats are able to include high proportions of heather in their diets, browsing on heathlands even with high pasture availability (Celaya et al., 2007). Osoro et al. (2007b) showed the limitation of these shrub species located in marginal lands with poor soils to develop sustainable ruminant production systems. However, the availability of these shrub species, together with areas of improved grass-legume pastures, could be an effective tool to achieve the sustainability of ruminant production systems and animal welfare in these marginal mountain areas (Celaya et al., 2008).

Recently, a number of studies have been conducted to investigate the anthelmintic and nutritional effect of heather supplementation in naturally infected grazing goats by GI nematodes (Table

1). Although Bahuaud et al. (2006) studied the effect of tannin extracts from *Erica erigena* on the exsheatment of third-stage infective larvae of *Haemonchus contortus* and *Trichostrongylus colubriformis*, to our knowledge, the anthelmintic activity of heather in goats was not addressed by other research teams. Therefore, this review focuses basically on our findings. These studies were carried out under farm conditions in a less favoured area, where grazing animals are extensively raised and included in the dynamics of a natural ecosystem. The trials compared the response to GI nematode infections and animal performance and nutrition in goats supplemented or not with heather. Interactions between heather availability and other alternative methods to control GI nematode infections based on grazing management (stocking rate) or nutrition (energy supply), as well as the potential adaptation of the rumen microbiota to the consumption of tannins, were also studied.

The experiments were conducted at "El Carbayal" research farm located in north-western Spain (lat. 43°21´N, long. 6°53´W; Sierra de San Isidro, Illano, Asturias). This is a mountain area of 900-1,000 m above sea level, with an average rainfall of 1550 mm/year, and where shrubby heather-gorse vegetation is dominant. Trials were established during the common grazing season in that area, which usually extended from late spring (May-June) to fall (October-November) depending on the climatic conditions. The studies were carried out between 2003 and 2007 on plots in which the vegetation had been improved in 2001 by ploughing the soil, dressing and sowing perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*), and removing any heather that was present.

Cashmere goats of different ages (does and kids) and physiological status (lactating and non-lactating) were used in the studies. The effect of heather administration to local Celtiberic non-lactating does was also investigated (Osoro et al., 2007a). All animals were reared outdoors under pasture conditions throughout the entire experimental period, without night confinement, and not excluding GI nematode infection. Before beginning the experiments, all goats were dosed orally with ivermectin (0,2 mg ivermectin/kg live weight, Oramec®, Merial), checking the efficacy of the anthelmintic treatment three weeks after administration by means of individual examination of FEC and inspection of clinical signs to confirm that goats did not show patent infection. Measurements included grass availability, botanical and chemical composition of pasture and heather, percentage of heather in the diet of each individual goat, ruminal fermentation parameters, body weight, body condition score, and parasitological studies (methods are described by Osoro et al., 2007c). Individual FECs and identification of infective third-stage larvae obtained by coprocultures were estimated in all animals at monthly or fortnightly intervals. In some experiments (Osoro et al., 2009), groups of goats of each treatment were slaughtered and adult worms were recovered from abomasums and intestines and counted in 10% aliquot samples. In order to assess the nematode development and fertility per female worm, the lengths of worms of each species were measured and the number of eggs *in utero* was directly counted, respectively. All experiments were carried out in accordance with Spanish Royal Decree 1201/2005 (based on European Council Directive 86/609/ECC) for the protection of animals used for experimental and other scientific purposes. Supplemented goats were offered freshly cut heather *ad libitum* twice per week in the morning during the grazing season. The heather was offered to animals in a cage in the field, and contained mainly *Calluna vulgaris*, *Erica umbellata* and *Erica cinerea*. *Erica tetralix* and *Daboecia cantabrica* were also present but in a lower (< 5%) percentage. The proportion of heather species in the supplement did not change significantly during the experimental periods. The nutritive quality of heather was lower than that of rye-grass-clover pasture (crude protein contents ranged from 55 to 71 g/kg DM in heather and from 78 to 209 g/kg DM in pasture), but no great differences were found over the grazing season despite a tendency to decrease as the season advanced. Total tannin contents in heather did not vary greatly during the grazing season and the mean content analysed by the Folin-Ciocalteu assay in combination with polyvinyl-polypyrrolidone (Makkar, 2003) and expressed as g tannic acid equivalents per kg dry matter

Table 1. Summary of the results of experiments with tannin-containing heather (Ericaceae) supplementation in grazing Cashmere goats.

Reference	Osoro et al. 2007a	Osoro et al. 2007c	Frutos et al. 2008	Osoro et al. 2009	Celaya et al. 2010	
Methods						
Goats	Adult non-lactating (including local Celtiberic) Young (< 1 year-old) females	Adult non-lactating	Adult lactating	Adult non-lactating	Period 1 Adult lactating	Period 2 Adult non-lactating
n	48	40	48	62	48	40
Experimental period	June-November	May-September	May-September	May-October	April-August	August-November
Experimental design	H+ vs. H-	4 treatments in a 2 x 2 factorial design: H+ and H- AT+ and AT-	H+ vs. H-	4 treatments in a 2 x 2 factorial design: H+ and H- HSR and LSR	H+ vs. H-	4 treatments in a 2 x 2 factorial design: H+ and H- O+ and O-
Heather in the diet (%)	ND	2-4 (June) 20-30 (September)	29 (August)	20 (August)	21 (June)	32 (October)
Tannin content in heather (g tannic acid equivalents/kg DM)	30-47	70-86	64	61-97	84	
Results						
FEC (%reduction)	Lower in H+ in August and September in adult goats (40%)	Interaction between H+ and AH+ (September) (70-80%)	Lower in H+ in August and September (30%)	Interaction between H+ and SR (15-30% in HSR)	Lower in H+ in July and August (50%)	Lower in H+. (42%) Lower in O+ (59%)
Worm burden	ND	ND	ND	Lower *T. circumcincta* counts in HSR *Trichostrongylus* spp. counts higher in HSR Lower development and fecundity in *Trichostrongylus* spp. in HSR	ND	ND
Body weight changes	Lower decrease in adult H+ goats	No effect	Lower decrease in adult H+ goats	Better changes in H+	Lower decrease in H+	Higher increase in H+ and O+
Body Condition score	ND	No effect	Lower decrease in adult H+ goats	No effect	Lower decrease in H+	Higher increase in O+
Ruminal fermentation parameters	ND	A: Lower in H+ VFA: Higher in H+	A: Lower in H+ VFA: Higher in H+	ND	A lower in H+	A lower in H+ and O+
Mortality			7 goats in H- Lower mortality rate in H+			

H+ = supplemented with heather; H- = non-supplemented with heather; AT+ = dosed with anthelmintic (ivermectin) every two months; AT- = not dosed with anthelmintic; HSR = high stocking rate (38 goats/ha); LSR = low stocking rate (24 goats/ha); O+ = supplemented with oats (0.5 kg fresh matter per head and day); O- = non-supplemented with oats; ND = not done; FEC = faecal egg count, A = ammonia concentration; VFA = volatile fatty acids

3. Effects of heather supplementation on parasitic nematodes

In the experimental conditions indicated above, the effects of heather supplementation were investigated on an established population of adult GI nematodes and indirectly on incoming third-stage larvae in goats at pasture. The presence of nematode eggs in faeces increased in all goats over the grazing season (May-June to October/November) and the greatest FECs were observed between September and November. Goats without heather supplement showed mean FECs of 5,000 to 8,000 eggs per gram of faeces (epg) with maximum numbers of 20,500 epg in mid-September (Osoro et al., 2007c). Nematode genera, identified on third-stage larvae obtained from coprocultures were *Teladorsagia circumcincta*, *H. contortus*, *Trichostrongylus* spp., *Oesophagostomum* spp., and *Chabertia ovina*. *Trichostrongylus* spp. were the GI nematodes more frequently identified over the whole experimental period and their percentages increased during the grazing period (from 20-30% in June to 70-90% in November). On the contrary, the percentages of *T. circumcincta* decreased from 50-70% in June to 5-10% in November. *Haemonchus contortus*, *Oesophagostomum* spp. and *Ch. ovina* were only detected, at low levels (less than 5%), after midsummer.

Adult goats supplemented with heather showed lower FEC than non-supplemented animals (Figure 1), and this effect was statistically significant between late August and the end of grazing season (Celaya et al., 2010; Frutos et al., 2008; Osoro et al., 2007a). Furthermore, a negative correlation between the percentage of heather in the diet and FEC in August and September was also found (Osoro et al., 2007c). The FEC reduction level in heather supplemented goats compared to control goats varied between 40% and 75%. No differences among nematode genera identified in coprocultures due to supplementation with heather were found.

The reduction in nematode egg excretion observed in goats supplemented with heather agrees with other studies involving supplementation of CT-containing plants to goats naturally infected by GI nematodes as mentioned before (Hoste et al., 2005a; Madibela and Jansen, 2003; Min et al., 2004, 2005; Moore et al., 2008; Paolini et al., 2003b, 2005b; Pomroy and Adlington, 2006; Shaik et al., 2006; Terrill et al., 2007, 2009). A rise in FECs has also been observed in naturally infected goats grazing acacia (8 different genera) shrub woodland and given polyethylene glycol (PEG), a tannin-binding agent that inhibits the effects of tannins (Kabasa et al., 2000).

The lower FEC could be of double origin, either as a consequence of a reduction in the nematode burden or a lower fecundity of female worms. Cashmere goats grazing upland perennial ryegrass-white clover pastures at two stocking rates (38 vs. 24 goats/ha) and supplemented or not with heather were necropsied at the end of the season (Osoro et al., 2009). In goats managed under the high stocking rate, the mean of total *T. circumcincta* counts was significantly lower in supplemented animals, and it is also noteworthy that fecundity *in utero* and length of *T. circumcincta* females in supplemented goats were higher than those observed in non-supplemented animals. This probably indicates a long-term established nematode population, suggesting that heather could affect the development of the incoming third-stage *T. circumcincta* larvae. On the contrary, no differences were found in *Trichostrongylus* spp. counts, but fecundity and development of female nematodes tended to be lower than observed in control goats. Lower adult *T. circumcincta* and *Trichostrongylus* spp. counts were also observed in grazing Cashmere does and their single kids supplemented with heather (unpublished data). The fertility of female trichostrongyles in those does tended to be lower when compared to controls. In kids, the fertility of abomasum trichostrongyles was significantly lower than controls, but no differences for intestinal nematodes were found.

Previous studies reported a variable effect of tannins on parasitic nematode numbers naturally-acquired after they have been established in goats. Under tropical conditions, a significant reduction in FECs (34%) and nematode numbers was found in goats experimentally infected with *H. contortus* which received dried leaves of *Acacia karoo*, a tropical

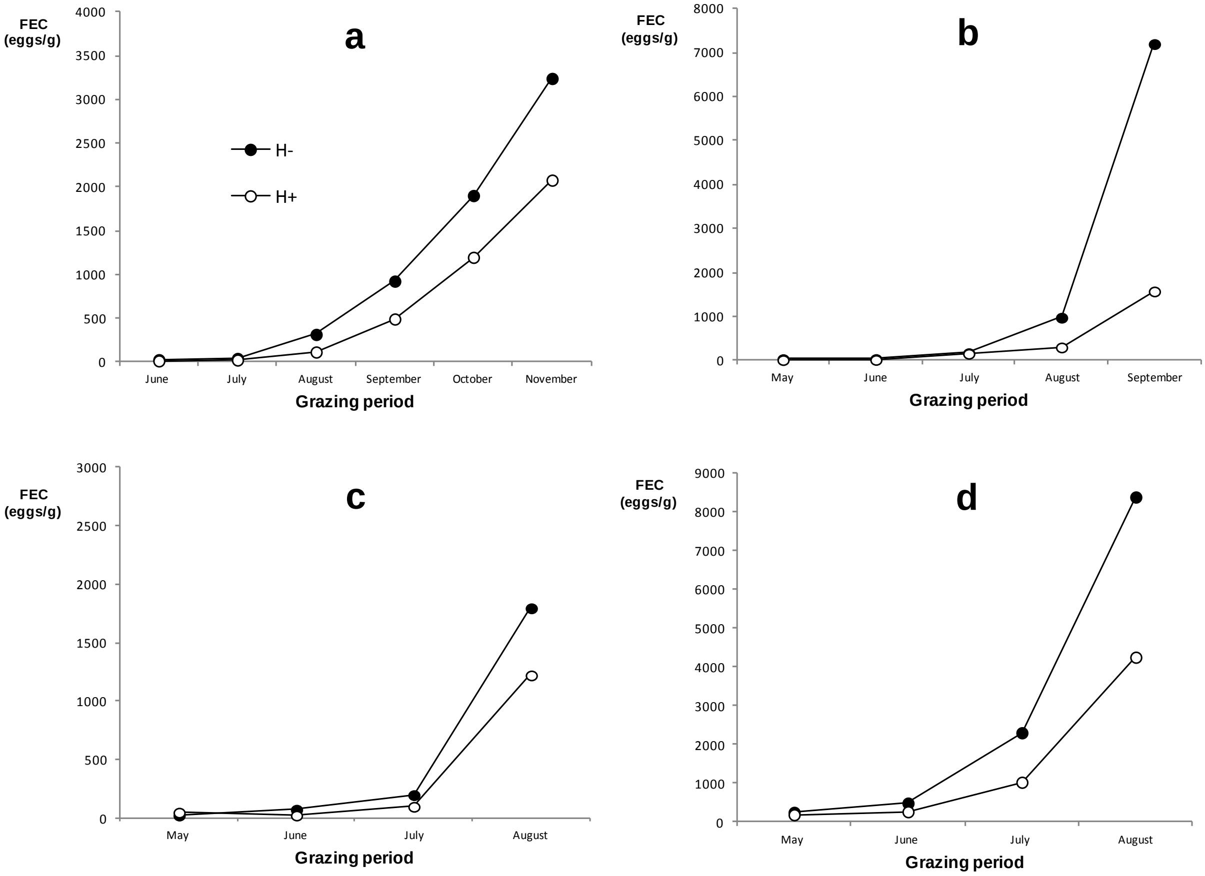

Figure 1. Faecal gastrointestinal nematode egg counts (FEC) during the experimental grazing period in nonlactating (a, b) and lactating (c, d) goats grazing perennial rye-grass-white clover pastures, with or without heather supplementation (H+, supplemented with freshly cut heather *ad libitum* every 3 days; H-, nonsupplemented). Data from (a) Osoro et al. (2007a), (b) Osoro et al. (2007c), (c) Frutos et al. (2008), and (d) Celaya et al. (2010)

leguminous tree rich in CT (Kahiya et al., 2003). The consumption of *Lysiloma latisiliquum*, a common tree species of the tropical forest vegetation of Central America has leaves which contain high levels of CT, was also studied in goats infected with *H. contortus* and *T. colubriformis*, showing a reduction in the establishment of both species larvae (Brunet et al., 2008b). The effects of sainfoin hay have also been tested on existing GI nematode parasite populations (Hoste et al., 2005a; Paolini et al., 2003b, 2005a) and incoming larvae of *H. contortus* (Paolini et al., 2005b). Goats naturally infected with a mixed infection of *H. contortus*, *T. circumcincta* and *T. colubriformis* and fed sainfoin showed lower levels of nematode egg excretion and a decrease in worm fertility but no change in worm population. In addition, a higher consumption of hay and better host resilience were observed (Paolini et al., 2005a). Another study carried out in a dairy goat farm showed that dairy goats naturally infected by GI nematodes and fed sainfoin regularly show a significant reduction of egg output, without negative consequences on milk production when compared with goats fed no sainfoin (Hoste et al., 2005a). However, there was no effect from sainfoin on the establishment of *H. contortus* third-stage larvae in experimentally infected goats (Paolini et al., 2005b).

The effects of a commercial extract of quebracho (*Schinopsis* sp.) CT have been investigated in goats under controlled conditions. These studies compared the effects of CT either on an established population of adult worms (curative treatment) or on incoming third-stage larvae (preventive treatment). In goats previously infected with 10,000 third-stage larvae of *H. contortus*, a significant egg output reduction with a decrease in female fecundity was observed (Paolini et al., 2003a). However, no effects of quebracho CT were found on worms established in goats experimentally infected with 1,500 larvae of *H. contortus* per day for three consecutive days (Paolini et al., 2005b). Similar experiments conducted to investigate the effects of quebracho CT in goats on adult populations of *T. circumcincta* and *T. colubriformis*, and the establishment of infective larvae of these two species showed a curative effect of CT on GI nematodes and a reduction by two thirds of the worm populations recovered at necropsy; this effect being significant for the intestinal species and close to significant for the abomasal one (Paolini et al., 2003c).

Sericea lespedeza is a high-CT warm season perennial legume, and when consumed by goats, is associated with reduced FEC of GI nematodes (Min et al., 2004). Goats fed sericea lespedeza and infected with *H. contortus* showed a significant reduction in FEC and increased packed cell volume compared with controls fed perennial warm-season grass (predominantly bermudagrass: *Cynodon dactylon*) (Shaik et al., 2006). In addition, there was a direct effect on adult worms, with significantly lower numbers of both abomasal (*H. contortus* and *T. circumcincta*) and intestinal (*T. colubriformis*) nematodes when compared with control goats (Shaik et al., 2006). In southern US conditions, goats fed sericea lespedeza hay (50-70% of the diet, Terrill et al., 2009) can effectively reduce nematode parasite infection levels through direct anthelmintic effects on the adult worms in the GI tract and by reducing parasite egg viability and/or larval development in faeces; and thus increase their performance (Moore et al., 2008; Shaik et al., 2006). Pelleting sericea lespedeza hay may enhance its efficacy against parasitic nematodes and facilitate the broader use of this forage in a small ruminant GI nematodes control program (Terrill et al., 2007).

In summary, CT could affect the establishment and consequently reduce the worm burden when acting on third stage-larvae. In contrast, they seem to mainly affect the worm reproductive function and the consequent contamination of pasture in animals harbouring adult worms. However, the mechanisms responsible for the effects of CT remain unclear. The reduction in FEC found in grazing goats cannot be attributed to the positive effect of extra protein availability for the animals supplemented with heather, because of the low protein content of heather (about 6.4%). Nevertheless, the possibility of increasing intestinal protein availability in ruminants, e.g. through preventing dietary protein degradation in the rumen due to the CT effect (Mueller-Harvey, 2006; Waghorn, 2008), could indirectly affect parasitism level and performance. Assuming a lower protein supply of heather-containing diet compared to only-pasture diet, the FEC reduction observed in heather supplemented goats suggests a direct effect of heather consumption on parasite burden. *In vivo* studies (e.g. Brunet et al., 2008b) suggest a direct effect of CT on GI nematodes and *in vitro* experiments showed that CT interfere with the two steps of the larval establishment, exsheathment (Brunet et al., 2007) and the mucosal penetration (Brunet et al., 2008a). A delay in *H. contortus* larvae exsheathment was observed when CT-*E. erigena* extracts (600 µg/mL) were tested *in vitro*, but no effect was observed on *T. colubriformis* larvae (Bahuaud et al., 2006). However, it is not possible to discern between the effects of heather secondary compounds per se and potential diet composition effects on faecal output and eggs recovery.

Recently, trials with Cashmere goats experimentally infected with *T. circumcincta* and *T. colubriformis* have been conducted to investigate the preventive (on incoming third-stage larvae) and curative (on adult patent nematode population) effects of heather supplementation under controlled conditions (Ferre et al., 2009; Moreno-Gonzalo et al., 2009). In the curative experiment, a group of goats infected with *T.*

colubriformis and supplemented with heather plus PEG was included (Frutos et al., in press). Initial results suggest that heather administration is associated with a reduction in the establishment of nematode larvae in the host, and a development and fecundity decrease of female parasite nematodes, but no reduction in worm numbers (Ferre et al., 2009; Moreno-Gonzalo et al., 2009).

4. Effects of heather supplementation on host performance and nutrition

In the grazing conditions described above, all adult Cashmere goats at pasture showed negative mean live weight changes for the whole experimental period, probably due to the limited availability of pasture (Merchant and Riach, 1994) and the increase in parasite burden (Coop and Kyriazakis, 2001) during the grazing season. Live weight decrease under low pasture availability conditions has been observed previously in goats grazing heather-gorse natural communities (Celaya et al., 2008). However, heather supplementation had a positive effect on live weight changes, and goats fed heather showed smaller losses of live body weight and body condition score than those with non-supplemented diets (Table 1). Furthermore, a positive correlation was observed between the percentage of heather in the diet and body weight changes during the grazing season (Osoro et al., 2007a, 2009). In contrast, as expected, the correlation coefficient between body weight changes and FEC was negative (Osoro et al., 2007c, 2009). The negative relationship between GI nematode infections and body weight changes has been observed in experimental and natural infections (Coop and Kyriazakis, 2001) and improved performance associated with the consumption of CT-rich plants, such as sericea lespedeza, was reported in Angora (Min et al., 2005) and Kiko x Spanish (Moore et al., 2008) goats naturally infected by GI nematodes.

Two studies were also conducted to investigate the interaction of feeding tannin-containing heather with grazing management (Osoro et al., 2009), and with the supplementation with an energy feed (Celaya et al., 2010), respectively. In the first experiment, goats were managed at two stocking rates (24 and 38 goats/ha), and in the second, some groups of goats received oats (*Avena sativa*) supplementation consisting in 0.5 kg fresh matter per head per day. Although a significant effect of the stocking rate on GIN was observed, no differences in FEC were detected between goats supplemented with heather and those non-supplemented. A lower infectious pressure on FEC basis and subsequent nematode burden when compared to previous experiments, probably caused by an anomalous dry year could explain these results. However, supplemented goats under low stocking rate gained more live weight and a positive correlation was found between the percentage of heather in the diet and body weight changes. The combination of heather and oats significantly improved animal performance and reduced the FEC in supplemented animals. These results confirm the improved response to GI nematode infections when host nutrition is improved by supplementary feeding (Hoste et al., 2005b). Grazing kids supplemented with barley (*Hordeum vulgare*) and fed *Duddingtonia flagrans* spores showed reduced FEC and GI nematode burdens (Gómez-Rincón et al., 2007). In addition, Gutierrez-Segura et al. (2002) found improved resilience and resistance in browsing Criollo kids fed an energy supplement such as maize (*Zea mays*) compared with non-supplemented animals.

The work described was conducted under practical conditions (Table 1), trying to simulate extensive production systems of a less-favoured area where availability of forage may be limited during the summer and a number of goats not dewormed in mid-Autumn may die. The supplementation with heather reduced the goat mortality rate (Frutos et al., 2008; Osoro et al., 2007c). This is consistent with previous studies reporting the positive effect of CT consumption on the resilience of goats naturally infected by GI nematodes grazing shrub/woodlands (Kabasa et al., 2000) or feeding sainfoin (Paolini et al., 2005b) or sericea lespedeza (Min et al., 2005; Moore et al., 2008).

In another study performed in a heath-gorse shrubland with 24% of improved perennial rye-grass-white clover pasture and managing lactating suckler cows, ewes, and goats under mixed grazing, goats showed higher increases in FEC over the grazing season than cattle or sheep (Celaya et al., 2008). In part, this is probably due to a lower level of immunity developed in goats as they are natural browsers, compared with cattle or sheep which are mainly grazers (Hoste et al., 2008). Nevertheless, in this study the FECs found in goats, with maximum values of 200 epg at the end of the grazing season, were lower than those found in goats grazing on rye-grass-white clover pastures with no available heather, with a maximum of 8,600 epg (Frutos et al., 2008; Osoro et al., 2007a, 2007c), which suggests that integrating both vegetation types could enhance goat performance because of improved nutrition and health status.

Regarding the nutritional studies, although erroneous generalizations have persisted since the first reviews in the 1960s and 1970s which stated that tannins were harmful or toxic to mammals (Mueller-Harvey, 2006), our results have shown that the amount of tannins ingested by grazing goats supplemented with heather was insufficient to exert substantial anti-nutritional effects (Frutos et al., 2008; Osoro et al., 2007a, 2007c). In fact, several authors have indicated that dietary concentrations of 50 g CT/kg DM are nutritionally beneficial (even though these recommendations originated mainly from feeding trials with *Lotus* species and cannot be directly applied to other feeds, Mueller-Harvey, 2006). The main benefit of tannins in ruminant nutrition stems from their negative effect on proteolysis and the initial consequence is a lower concentration of ammonia in the ruminal fluid, which was commonly observed in the heather supplemented animals. On the other hand, the volatile fatty acid concentrations were greater in these animals, which would point to an adaptation of rumen microorganisms to the consumption of CT-containing heather and a subsequently improved efficiency of ruminal fermentation (Table 1, Celaya et al., 2010; Frutos et al., 2008; Osoro et al., 2007c). The experimental infections using PEG showed the existence of some mild detrimental effects of heather tannins on diet digestibility, but they were not as negative as those observed previously using other CT-containing plant species (Frutos et al., in press). *In vitro* experiments involving batch cultures of rumen microorganisms, used to further investigate the nutritional effects of the inclusion of heather in the diet, confirmed the *in vivo* observations (Frutos et al., 2008 and in press).

Anthelmintic effects (FEC reduction) were achieved with the dietary incorporation of estimated 20-32% heather containing up to 8.6% tannins, which may be considered as a low-to-moderate intake of tannins. Although the lowest dietary tannin threshold needed for anthelmintic effects remains unclear, results from the indoor experiments using PEG suggest that this is probably quite low and that the proportion of heather included in the diet to this end could be lowered (Frutos et al., in press). In addition, it is worth mentioning that the notion “low-to-moderate” tannin content could be misleading because, first, different bioactive plants contain tannins with different structures and hence reactivities, and second, different methods are used to analyse phenolic compounds and the equivalents in which the tannin content are expressed are not always given.

5. Conclusions and perspectives

It is concluded that (i) heather supplementation in grazing goats significantly reduce the level of GI nematode egg excretion, (ii) the faecal nematode egg count reduction could be associated with a decrease in worm fertility and/or reduction in the establishment of incoming third-stage larvae, (iii) consumption of heather is associated with an apparent greater resilience of goats to GI nematode infections, and (iv) the amount of tannins consumed by goats offered heather does not seem to be associated with substantial anti-nutritional effects, which eventually resulted in a better performance of animals incorporating these shrubs in their diet.

In the near future, the characterization of bioactive compounds included in heather and *in vitro* studies to clarify their mechanism of action on different parasite stages (e.g. the possible interference on larval exsheathment or mucosal penetration, or both, to explain the reduction in the establishment of nematode larvae) will be carried out. Furthermore, trials including different herbivore species, such as goats, sheep, cattle, and horses, have been designed to investigate the effects of mixed grazing partially improved heathlands on GI nematodes infections and performance.

Practical application of this knowledge in temperate areas would support the management of plots integrating productively improved pastures with high nutritive value (rye-grass-white clover) with natural vegetation communities including tannin-containing plants, such as heather species. Improved pastures could be used to increase protein and energy intake and natural vegetation for fibre, minerals, and secondary compounds that contribute to achieve sustainable goat (and other herbivores) production systems with a lower dependence on conventional chemotherapy.

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References

Bahuaud, D., Martínez-Ortíz de Montellano, C., Chauveau, S., Prevot, F., Torres-Acosta, F., Fouraste, I., Hoste, H., 2006. Effects of four tanniferous plant extracts on the in vitro exsheatment of third-stage larvae of parasitic nematodes. Parasitology 132, 545-554.

Brunet, S., Aufrere, J., El Babili, F., Fouraste, I., Hoste, H., 2007. The kinetics of exsheathment of infective nematode larvae is disturbed in presence of tannin-rich plant (sainfoin) both in vitro and in vivo. Parasitology 134, 1253-1262.

Brunet, S., Jackson, F., Hoste, H., 2008a. Effects of sainfoin (*Onobrychis viciifolia*) extract and monomers of condensed tannins on the association of abomasal nematode larvae with fundic explants. Int. J. parasitol. 38, 783-790.

Brunet, S., Martínez-Ortíz de Montellano, C., Torres-Acosta, J.F.J., Sandoval-Castro, C.A., Aguilar-Caballero, A.J., Capetillo-Leal, C., Hoste, H., 2008b. Effect of the consumption of *Lysiloma latisiliquum* on the larval establishment of gastrointestinal nematodes in goats. Vet. Parasitol. 157, 81-88.

Celaya, R., Oliván, M., Ferreira, L.M.M., Martínez, A., García, U., Osoro, K., 2007. Comparison of grazing behaviour, dietary overlap and performance in non-lactating domestic ruminants grazing on marginal heathland areas. Livest. Sci. 106, 271-281.

Celaya, R., Benavides, R., García, U., Ferreira, L.M.M., Ferre, I., Martínez, A., Ortega-Mora, L.M., Osoro, K., 2008. Grazing behaviour and performance of lactating suckler cows, ewes and goats on partially improved heathlands. Animal 2, 1818-1831.

Celaya, R., Ferreira, L.M.M., Moreno-Gonzalo, J., Frutos, P., Hervás, G., Ferre, I., García, U., Ortega-Mora, L.M., Osoro, K., 2010. Effects of heather and oat supplementation on gastrointestinal nematode infections and performance of grazing Cashmere goats. Small Rumin. Res. 91, 186-192.

Cheeke, P.R., Palo, R.T., 1995. Plant toxins and mammalian herbivores: co-evolutionary relationships and antinutritional effects. In: Journet, M., Grenet, E., Farce, M.H., Thériez, M., Demarquilly, C. (Eds.). Recent Developments in the Nutrition of Herbivores. Proceedings of the IVth International Symposium on the Nutrition of Herbivores. INRA Editions, Paris, France, pp. 437-456.

Coop, R.L., Kyriazakis, I., 2001. Influence of host nutrition on the development and consequences of nematode parasitism in ruminants. Trends Parasitol. 17, 325-330.

Ferre, I., Moreno-Gonzalo, J., García, U., Frutos, P., Celaya, R., Ferreira, L.M.M., Ortega-Mora, L.M., Osoro, K., 2009. Anthelmintic effect of heather supplementation on gastrointestinal nematodes in goats. In: Proceedings of the COST Action FA0805 Goat-parasite interactions: from knowledge to control (CAPARA), 1st working groups workshop & Management Committee Meeting, Thessaloniki, Greece.

Frutos, P., Hervás, G., Ramos, G., Giráldez, F.J., Mantecón, A.R., 2002. Condensed tannin content of several shrub species from a mountain area in northern Spain and its relationship to various indicators of nutritive value. Anim. Feed Sci. Technol. 95, 215-226.

Frutos, P., Moreno-Gonzalo, J., Hervás, G., García, U., Ferreira, L.M.M., Celaya, R., Toral, P.G., Ortega-Mora, L.M., Ferre, I., Osoro, K., 2008. Is the anthelmintic effect of heather supplementation to grazing goats always accompanied by anti-nutritional effects? Animal 2, 1449-1456.

Frutos, P., Hervás, G., Celaya, R., Moreno-Gonzalo, J., Rodríguez, A.B., García, U., Ferre, I., Ortega-Mora, L.M., Osoro, K. Effect of the use of heather as an anthelmintic in goats infected with *Trichostrongylus colubriformis* on ruminal fermentation and digestibility. Options Méditerranéennes (in press).

Gómez-Rincón, C., Uriarte, J., Valderrábano, J., 2007. Effect of nematophagous fungus *Duddingtonia flagrans* and energy supplementation on the epidemiology of naturally infected kids. Vet. Res. 38, 141-150.

Gutierrez-Segura, I., Torres-Acosta, J.F., Aguilar-Caballero, A., Canul-Ku, I., Lozano-Argaez, I., Cob-Galera, L., 2002. Improving resilience and resistance against nematode infections through maize supplementation in Criollo kids. In: Responding to the increasing Global Demand for Animal Products. BSAS-ASAS, Mérida, Yucatán, México, pp. 78-79.

Hoste, H., Gaillard, L., Le Frileux, Y., 2005a. Consequences of the regular distribution of sainfoin hay on gastrointestinal parasitism with nematodes and milk production in dairy goats. Vet. Parasitol. 59, 265-271.

Hoste, H., Torres-Acosta, J.F., Paolini, V., Aguilar-Caballero, A., Etter, E., Lefrileux, Y., Chartier, C., Broqua, C., 2005b. Interactions between nutrition and gastrointestinal infections with parasitic nematodes in goats. Small Rumin. Res. 60, 141-151.

Hoste, H., Jackson, F., Athanasiadou, S., Thamsborg, S.M., Hoskin, S.O., 2006. The effects of tannin-rich plants on parasitic nematodes in ruminants. Trends Parasitol. 22, 253-261.

Hoste, H., Torres-Acosta, J.F.J., Aguilar-Caballero, A.J., 2008. Nutrition-parasite interactions in goats: is immunoregulation involved in the control of gastrointestinal nematodes? Parasite Immunol. 30, 79-88.

Kabasa, J.D., Opuda-Asibo, J., ter Meulen, U., 2000. The effect of oral administration of polyethylene glycol on faecal helminth egg counts in pregnant goats grazed on browse containing condensed tannins. Trop. Anim. Health Prod. 32, 73-86.

Kahiya, C., Mukaratirwa, S., Thamsborg, S.M., 2003. Effects of *Acacia nilotica* and *Acacia karoo* diets on *Haemonchus contortus* infection in goats. Vet. Parasitol. 115, 265-274.

Kaplan, R.M., 2004. Drug resistance in nematodes of veterinary importance: a status report. Trends Parasitol. 20, 477-481.

Ketzis, J.K., Vercruysse, J., Stromberg, B.E., Larsen, M., Athanasiadou, S., Houdijk, J.G.M., 2006. Evaluation of efficacy expectations for novel and non-chemical helminth control strategies in ruminants. Vet. Parasitol. 139, 321-335.

Madibela, O.R., Jansen, K., 2003. The use of indigenous parasitic plant (*Viscum verrocosum*) in reducing faecal egg counts in female Tswana goats. Liv. Res. Rural Dev. 15, 1-6.

Makkar, H.P., 2003. Quantification of Tannins in Tree and Shrub Foliage. A Laboratory Manual. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Merchant, M., Riach, D.J., 1994. The intake and performance of Cashmere goats grazing sown swards. Grass Forage Sci. 49, 429-437.

Min, B.R., Pomroy, W.E., Hart, S.P., Saúl, T., 2004. The effect of short-term consumption of a forage containing condensed tannins on gastro-intestinal nematode parasite infections in grazing wether goats. Small Rumin. Res. 51, 279-283.

Min, B.R., Hart, S.P., Millar, D., Tomita, G.M., Loetz, E., Saúl, T., 2005. The effect of grazing forage containing condensed tannins on gastro-intestinal parasite infection and milk composition in Angora does. Vet. Parasitol. 130, 105-113.

Moreno-Gonzalo, J., Osoro, K., García, U., Frutos, P., Celaya, R., Ferreira, L.M.M., Ortega-Mora, L.M., Ferre, I., 2009. Effect of heather consumption on incoming larvae and established population of *Trichostrongylus colubriformis* in experimentally infected Cashmere goats. In: Proceedings of the 22nd International Conference of the World Association for the Advancement of Veterinary Parasitology, Calgary, Canada.

Moore, D.A., Terrill, T.H., Kouakou, B., Shaik, S.A., Mosjidis, J.A., Miller, J.E., Vanguru, M., Kannan, G., Burke, J.M., 2008. The effects of feeding sericea lespedeza hay on growth rate of goats naturally infected with gastrointestinal nematodes. J. Anim. Sci. 86, 2328-2337.

Mueller-Harvey, I., 2006. Unravelling the conundrum of tannins in animal nutrition and health. J. Sci. Food Agric. 86, 2010-2037.

Osoro, K., Benito-Peña, A., Frutos, P., García, U., Ortega-Mora, L.M., Celaya, R., Ferre, I., 2007a. The effect of heather supplementation on gastrointestinal nematode infections and performance in Cashmere and local Celtiberic goats on pasture. Small Rumin. Res. 67, 184-191.

Osoro, K., García, U., Jáuregi, B.M., Ferreira, L.M.M., Rook, A.J., Celaya, R., 2007b. Diet selection and live-weight changes of two breeds of goats grazing on heathlands. Animal 1, 449-457.

Osoro, K., Mateos-Sanz, A., Frutos, P., García, U., Ortega-Mora, L.M., Ferreira, L.M.M., Celaya, R., Ferre, I., 2007c. Anthelmintic and nutritional effects of heather supplementation on Cashmere goats grazing perennial ryegrass-white clover pastures. J. Anim. Sci. 85, 861-870.

Osoro, K., Celaya, R., Moreno-Gonzalo, J., Ferreira, L.M.M., García, U., Frutos, P., Ortega-Mora, L.M., Ferre, I., 2009. Effects of stocking rate and heather supplementation on gastrointestinal nematode infections and host performance in naturally infected Cashmere goats. Rangeland Ecol. Manage. 62, 127-135.

Paolini, V., Bergeaud, J.P., Grisez, C., Prevot, F., Dorchies, Ph., Hoste, H., 2003a. Effects of condensed tannins on goats experimentally infected with *Haemonchus contortus*. Vet. Parasitol. 113, 253-261.

Paolini, V., Dorchies, Ph., Hoste, H., 2003b. Effects of sainfoin hay on gastrointestinal nematode infections in goats. Vet. Rec. 152, 600-601.

Paolini, V., Frayssines, A., De La Farge, F., Dorchies, Ph., Hoste, H., 2003c. Effects of condensed tannins on established populations and on incoming larvae of *Trichostrongylus colubriformis* and *Teladorsagia circumcincta* in goats. Vet. Res. 34, 331-339.

Paolini, V., De La Farge, F., Prevot, F., Dorchies, Ph., Hoste, H., 2005a. Effects of the repeated distribution of sainfoin hay on the resistance and the resilience of goats naturally infected with gastrointestinal nematodes. Vet. Parasitol. 127, 277-283.

Paolini, V., Prevot, F., Dorchies, Ph., Hoste, H., 2005b. Lack of effects of quebracho and sainfoin hay on incoming third-stage larvae of *Haemonchus contortus* in goats. Vet. J. 170, 260-263.

Pomroy, W.E., Adlington, B.A., 2006. Efficacy of short term feeding of sulla (*Hedysarum coronarium*) to young goats against a mixed burden of gastrointestinal nematodes. Vet. Parasitol. 136, 363-366.

Shaik, S.A., Terrill, T.H., Millar, J.E., Kouakou, B., Kankan, G., Kaplan, R.M., Burke, J.M., Mosjidis, J.A., 2006. Sericea lespedeza hay as a natural deworming agent against gastrointestinal nematode infections in goats. Vet. Parasitol. 139, 150-157.

Stear, M.J., Doligaska, M., Donskow-Schmelter, K, 2007. Alternatives to anthelmintics for the control of nematodes in livestock. Parasitology 134, 139-151.

Terrill, T.H., Mosjidis, J.A., Moore, D.A., Shaik, S.A., Millar, J.E., Burke, J.M., Muir, J.P., Wolfe, R., 2007. Effect of pelleting on efficacy of sericea lespedeza hay as a natural dewormer in goats. Vet. Parasitol. 146, 117-122.

Terrill, T.H., Dykes, G.S., Shaik, S.A., Miller, J.E., Kouakou, B., Kannan, G., Burke, J.M., Mosjidis, J.A., 2009. Efficacy of sericea lespedeza hay as a natural dewormer in goats: Dose titration study. Vet. Parasitol. 163, 52-56.

Waghorn, G., 2008. Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat production - Progress and challenges. Anim. Feed Sci. Techol. 147, 116-139.

Waller, P.J., Thamsborg, S.M., 2004. Nematode control in “green” ruminant production systems. Trends Parasitol. 20, 493-497.

Justificación y objetivos

El censo caprino mundial es aproximadamente 867 millones de cabezas (FAOSTAT, 2010), de las cuales 13,1 millones se encuentran en la Unión Europea (EUROSTAT, 2011). España, con 2,7 millones de cabras, es el segundo país en cuanto al número de animales, por detrás de Grecia con 4,8 millones y por delante de Francia con 1,4 millones (EUROSTAT, 2011). La mayor parte del censo caprino se encuentra en países en vías de desarrollo en los que su importancia es más cualitativa que cuantitativa y está orientado a la producción de carne y leche para autoconsumo. Sin embargo, en los países industrializados la producción está dirigida a la producción de leche y quesos artesanales de gran valor comercial, en algunos casos de forma intensiva y, en otros, con una tendencia a la transformación en ganadería ecológica (Hoste et al., 2011b). En cualquier caso, el aprovechamiento de los recursos naturales y, por tanto, la cría de ganado caprino en sistemas extensivos o semiextensivos, parece ser la tendencia general en todo el mundo.

La explotación de rumiantes en pastoreo está asociada a las parasitosis y especialmente a las nematodosis gastrointestinales (Sykes, 1994). Las pérdidas económicas causadas por estas, que incluyen la disminución de rendimiento, aumento de los costes derivados de la administración de fármacos e incluso la mortalidad de animales comprometen la rentabilidad y viabilidad de las explotaciones ganaderas, especialmente aquellas de pequeños rumiantes (McLeod, 1995; Nieuwhof y Bishop, 2005). Por ello, se considera que las nematodosis gastrointestinales constituyen la parasitosis más importante de los pequeños rumiantes en pastoreo en todo el mundo (Perry y Randolph, 1999; Perry et al., 2002). Aunque el ganado ovino y caprino se encuentran parasitados por las mismas especies, se sospecha de cepas adaptadas al ganado caprino (Gasnier y Cabaret, 1996). Debe destacarse que el ganado caprino presenta importantes diferencias respecto al comportamiento en pastoreo con el ganado ovino y bovino. El caprino se comporta como ramoneador (*browser*) mientras que el ovino y bovino son considerados pacedores (*grazers*). Este comportamiento evita, en gran medida, el contacto de las cabras con las larvas infectantes de los nematodos gastrointestinales y, además, facilita el acceso a numerosas plantas que incluyen metabolitos secundarios. Este comportamiento también explicaría por qué las cabras han desarrollado escasa capacidad para desarrollar una respuesta inmune efectiva frente a estos parásitos, pero una mayor capacidad para evitar la intoxicación con sustancias exógenas (Hoste et al., 2008).

La aparición de cepas de nematodos resistentes a las principales familias de fármacos antihelmínticos (Kaplan, 2004; Jackson y Coop, 2000), especialmente en los pequeños rumiantes (Jabbar et al., 2006; Jackson et al., 2012), la necesidad de mantener periodos de supresión tras la administración de la mayoría de dichos fármacos y la demanda, cada vez mayor, por parte del consumidor de productos libres de cualquier tipo de residuo químico y respetuosos con el ambiente, han estimulado la búsqueda de métodos alternativos de control frente a estas nematodosis (Waller y Thamsborg, 2004; Ketsis et al., 2006; Hoste y Torres-Acosta, 2011a). Entre ellos, uno de los más estudiados es la utilización de nutracéuticos (alimentos considerados más por sus efectos beneficiosos sobre la salud que por su contribución a la nutrición del animal que los consume) que forman parte del pasto o se usan como forraje (las más estudiadas son aquellas que contienen taninos condensados), para el control de estas parasitosis (Hoste et al., 2006). Su uso permitiría un control rentable de los procesos parasitarios, sin periodos de supresión, con productos totalmente naturales y también beneficiosos desde el punto de vista nutritivo (Ramírez-Restrepo y Barry, 2005; Athanasiadou y Kyriazakis, 2004; Ketsis et al., 2006; Athanasiadou et al., 2007).

Aunque la mayoría de estudios se han llevado a cabo con plantas tropicales, también se ha investigado el efecto antihelmíntico de especies vegetales propias de climas templados, como los realizados por Shaik et al. (2006) con sericea lespedeza (*Lespedeza cuneata*), Paolini et al. (2005) con esparceta (*Onobrychis viciifolia*), Pomroy y Adlington (2006) con zulla (*Hedysarum coronarium*) y Moreno-Gonzalo et al. (2012) con brezo (especies pertenecientes a la familia Ericaceae).

El estudio del efecto antihelmíntico de una planta o conjunto de ellas se puede abordar desde dos perspectivas diferentes. La primera sería estudiar la actividad antihelmíntica de la planta o sus extractos frente a una o varias especies de parásitos mediante experimentos *in vitro* en cierta medida normalizados (Jackson y Hoste, 2010; Kotze et al., 2009). Si los resultados son satisfactorios, en segundo lugar se realizarían pruebas *in vivo* con el fin de llegar a una aplicación práctica (Manolaraki et al., 2010; Terrill et al., 2007, 2009). La segunda perspectiva sería seguir el camino inverso, es decir, comprobar mediante pruebas de campo que la planta estudiada es eficaz frente a las parasitosis y no posee efectos adversos, para después investigar en condiciones controladas y con experimentos *in vitro* sobre qué especie o estadio parasitario actúa, o cuáles son los componentes que lo provocan para poder optimizar su uso. Esta última opción es la utilizada en el trabajo que desarrollamos a continuación.

El objetivo general de la presente tesis doctoral fue estudiar el efecto antihelmíntico y nutritivo del brezo en el ganado caprino. En primer lugar, se estudió el efecto de la suplementación con brezo en ganado caprino en pastoreo. Después, se investigó el efecto antihelmíntico del consumo de brezo en cabras infectadas experimentalmente con *Trichostrongylus colubriformis y Teladorsagia circumcincta,* las dos especies de nematodos digestivos con mayor importancia en el ganado caprino de climas templados (Valcárcel y Romero., 1999; Osoro et al., 2007a,b; Celaya et al., 2010). Por último, se estudió mediante pruebas *in vitro* el efecto directo de extractos de brezo sobre varios estadios de desarrollo de las especies mencionadas. Para ello, se establecieron tres objetivos específicos.

Objetivo 1. Estudio del efecto antihelmíntico y nutritivo del consumo de brezo en cabras en pastoreo.

En este objetivo, desarrollado en el capítulo II de la tesis, se pretendió conocer el efecto del brezo sobre las nematodosis gastrointestinales de cabras en pastoreo cuando su disponibilidad es máxima. En experimentos previos ya se había observado una tendencia a la reducción de la intensidad parasitaria y mayor ganancia de peso en las cabras en pastoreo cuando se incrementaba el acceso a vegetación natural arbustiva (Osoro et al., 1995). Para ello se diseñaron dos experimentos. En el primero se estudió el efecto de la suplementación con brezo y la carga ganadera sobre parámetros parasitológicos (excreción fecal de huevos de nematodos gastrointestinales, intensidad parasitaria, especies implicadas y desarrollo y fecundidad de los parásitos) y sobre parámetros productivos (peso vivo, condición corporal y tasa de mortalidad). En el segundo experimento se estudió la cantidad de brezo que consumía voluntariamente el ganado caprino cuando tenía total disponibilidad y su efecto sobre algunos parámetros parasitológicos (excreción fecal de huevos), los parámetros productivos ya mencionados y nutricionales (producción total de gas, amonio y ácidos grasos volátiles), así como el estudio *in vitro* de la fermentación ruminal

Objetivo 2. Estudio del efecto antihelmíntico del consumo de brezo en cabras infectadas experimentalmente *por Trichostrongylus colubriformis* y *Teladorsagia circumcincta.*

El principal problema de los experimentos en pastoreo es la imposibilidad de conocer en todo momento la carga parasitaria de cada animal, ya que estos se infectan de manera gradual y con dosis discontinuas no predecibles aunque se investigue la carga parasitaria en la hierba. Sin embargo, al infectar experimentalmente a todos los animales con la misma dosis, la mayor o menor intensidad parasitaria no sería atribuible a una mayor o menor ingestión de larvas infectantes, hecho que puede ocurrir en infecciones naturales. Por ello, y para tener un adecuado control de los parámetros estudiados anteriormente en los experimentos en pastoreo se llevaron a cabo dos experimentos en condiciones controladas que se desarrollan en el capítulo III de esta tesis. En el primero se estudió el efecto antihelmíntico del consumo de brezo en cabras infectadas experimentalmente con *T. colubriformis*. Primero se investigó su efecto preventivo (el animal se encontraba consumiendo brezo cuando se produjo la infección) sobre la tasa de implantación y el desarrollo parasitario del nematodo. A continuación, se investigó el efecto de su consumo de forma curativa (el animal tenía una infección patente cuando empezó a consumir brezo) sobre la intensidad parasitaria, la excreción fecal de huevos y el desarrollo parasitario del nematodo. Además, a uno de los grupos de animales que consumió brezo en el experimento curativo se le administró polietilenglicol (PEG), un inhibidor de los taninos condensados, con el fin de dilucidar si el posible efecto antihelmíntico del brezo se debía o no a estos compuestos. En el segundo experimento se investigó el efecto antihelmíntico del consumo de brezo en cabras infectadas experimentalmente con *T. circumcincta*. De la misma forma que en el experimento anterior se estudió el efecto del consumo de brezo de forma preventiva y curativa.

Objetivo 3. Estudio del efecto *in vitro* de extractos de brezo sobre diferentes estadios parasitarios de *Trichostrongylus colubriformis*, *Teladorsagia circumcincta* y *Haemonchus contortus*.

En nuestro caso, los experimentos *in vitro* tienen el objetivo de poner en contacto un extracto bruto (que contiene taninos y otros compuestos fenólicos) o un extracto purificado (que contiene solo taninos condensados) de brezo con el parásito diana. Con estos experimentos se pretende evitar la influencia del hospedador en el efecto del brezo sobre el parásito, pero siempre intentando reproducir lo que sucede *in vivo*. Este procedimiento permite seleccionar la planta o sustancia extraída de la planta a estudiar y la especie y fase parasitaria de una forma más rápida y barata y en condiciones totalmente controladas. En los experimentos realizados y que se desarrollan en el capítulo IV de esta tesis, se utilizaron extractos de tres especies de brezo (*Calluna vulgaris*, *Erica cinerea* y *Erica umbellata*) y un cuarto extracto compuesto por una mezcla de las tres especies. Se realizaron tres ensayos para estudiar el efecto de los extractos sobre diferentes fases parasitarias. Para estudiar el efecto de los extractos de brezo sobre la eclosión de los huevos se realizó un ensayo de eclosión de huevos (*Egg Hatching Assay*). Para estudiar el efecto de los extractos de brezo sobre el desenvainamiento de las larvas de tercer estadio se realizó un ensayo de desenvainamiento larvario (*Larval Exsheathment Inhibition Assay*). Y, por último, para investigar el efecto de los extractos de brezo sobre la mortalidad de los vermes adultos se realizó un ensayo de inhibición de la motilidad de vermes adultos (*Adult Motility Inhibition Assay*). Tanto la eclosión de los huevos como el desenvainamiento de las larvas infectantes son procesos clave en la continuidad del ciclo biológico de los nematodos gastrointestinales, por ello tienen gran relevancia respecto al control de estas parasitosis.

Justification and objectives

The world goat population is approximately 867 million head (FAOSTAT, 2010), of which 13.1 million are in the European Union (EUROSTAT, 2011). Spain, with 2.7 million goats, has the second most amount of the animal, behind Greece with 4.8 million and ahead of France with 1.4 million (EUROSTAT, 2011). Most of the goat population is found in developing countries where its importance is more qualitative than quantitative and it is oriented to the production of meat and milk for personal consumption. However, in industrialized countries, the production is directed to the production of milk and cheese of great commercial value, in some cases with intensive farming and, in others, with a tendency to transform into organic farming (Hoste et al. 2011). In any case, the use of natural resources and, therefore, goat rearing in extensive or semi-extensive systems seems to be the general trend worldwide.

The exploitation of grazing ruminants is associated with parasitic diseases and especially with gastrointestinal nematode infections (Sykes, 1994). The economic losses caused by these diseases, including decreased performance, increased costs derived from administering drugs and even animal mortality, all compromise the profitability and viability of farms, especially those of small ruminants (McLeod, 1995; Nieuwhof and Bishop, 2005). Therefore, gastrointestinal nematode infections are considered the most important disease in grazing small ruminants worldwide (Perry and Randolph, 1999; Perry et al., 2002). Although sheep and goats are parasitized by the same species, it is suspected that goats have adapted strains (Gasnier, 1996). It should be noted that goats present important differences in grazing behavior from sheep and cattle. Goats are considered browsers while the sheep and cattle are considered grazers. This behavior avoids, largely, the contact of goats with the infective larvae of gastrointestinal nematodes and also facilitates their access to numerous plants including secondary metabolites. This behavior also explains why goats have developed little capacity to develop an effective immune response against these parasites, but a greater ability to avoid intoxication by exogenous substances (Hoste et al., 2008).

The emergence of resistant strains of nematodes to the major anthelmintic drug families (Kaplan, 2004; Jackson and Coop, 2000), especially in small ruminants (Jabbar et al., 2006, Jackson et al., 2012), the need to maintain suppression periods after the administration of most of these drugs and increasing demand by the consumer of products free of any chemical residue and friendly with the environment have stimulated the search for alternative control methods against these nematodiasis (Waller, 2004; Ketsis et al., 2006, Torres-Acosta and Hoste, 2011). Among them, one of the most studied is the use of nutraceuticals (foods considered more for their beneficial effects on health than for their contribution to the nutrition of the animal that consumes them) that are part of pasture or used as forage (most studied are those containing condensed tannins) for control of these parasites (Hoste et al., 2006). Its use would allow a cost-effective control of parasitic processes, without suppression periods, with completely natural products that are also beneficial from the nutritional point of view (Ramirez-Restrepo and Barry, 2005; Athanasiadou and Kyriazakis, 2004; Ketsis et al. 2006; Athanasiadou et al., 2007).

Although most of the studies have been carried out with tropical plants, the anthelmintic effect of plant species in temperate climates has been also studied by Shaik et al. (2006) with sericea lespedeza (*Lespedeza cuneata*), Paolini et al. (2005) with sainfoin (*Onobrychis viciifolia*), Pomroy et al. (2006) with sulla (*Hedysarum coronarium*) and Moreno-Gonzalo et al. (2012) with heather (species belonging to the Ericaceae family).

The study of the anthelmintic effect of a plant or group of them can be approached from two different perspectives. The first would be to study the anthelmintic activity of a plant or its extracts against one or more parasite species using *in vitro* experiments standardized to some extent (and Hoste Jackson, 2010; Kotze et al., 2009). If the results are satisfactory, second *in vivo* tests would be conducted in order to reach a practical application (Manolaraki et al., 2010; Terrill et al., 2007, 2009). The second approach would be to follow the reverse way, i.e. check through field trials if the plant is effective against parasites and has no adverse effects, and then investigate under controlled conditions and *in vitro* experiments on which species or parasitic stage acts, or what are the components that cause it to optimize its use. This last option is the one used in the work we develop below.

The overall objective of this thesis was to study the anthelmintic and nutritional effect of heather in goats. First, we studied the effect of heather supplementation in grazing goats. Next, we investigated the anthelmintic effect of heather consumption in goats experimentally infected with *Trichostrongylus colubriformis* and *Teladorsagia circumcincta*, the two most important nematode species in goats in temperate climates (Valcárcel y Romero., 1999; Osoro et al., 2007a, b; Celaya et al., 2010). Finally, the direct effect of heather extracts on various life stages of the mentioned species was studied *in vitro*. To achieve this, three specific objectives were established.

Objective 1. Study of the anthelmintic and nutritional effect of heather consumption in grazing goats.

In this objective, developed in the chapter II of this thesis, we attempted to determine the effect of heather consumption on the gastrointestinal nematode infections in grazing goats with total availability of heather. In previous experiments, a trend to reduce the parasite burden and greater weight gain in grazing goats was observed when the access to natural shrub vegetation was increased (Osoro et al., 1995). For this purpose two experiments were designed. In the first, we studied the effect of supplementation with heather and stocking rate on parasitological parameters (fecal egg excretion of gastrointestinal nematodes, worm burden, involved species and the development and fecundity of the parasites) and on production parameters (live weight, body condition and mortality). In the second experiment we studied the amount of heather voluntarily consumed by goats when they had total availability, and its effect on some parasitological parameters (fecal egg excretion), the production parameters above mentioned and on nutrition (total production of gas, ammonia and volatile fatty acids). The *in vitro* rumen fermentation was also studied

Objective 2. Study of the anthelmintic effect of heather in goats experimentally infected with *Trichostrongylus colubriformis* and *Teladorsagia circumcincta*.

The main problem of field experiments is the impossibility of knowing the worm burden of each animal as they gradually become infected and with discontinuous and unpredictable doses, even if the parasite burden in the grass is studied. However, if all animals are experimentally infected with the same dose, and the level of parasitic burden would not be attributable to a greater or lesser ingestion of infective larvae, which may occur in natural infections. For this reason, and in order to keep control of the parameters studied before in the grazing experiments, two experiments in controlled conditions that are developed in Chapter III of this thesis were carried out. In the first, the anthelmintic effect of heather consumption in goats experimentally infected with *T. colubriformis* was studied. First, we investigated the preventive effect (the animals were eating heather when the infection occurred) on the establishment rate and development of the nematode parasites. Then, we investigated the curative effect (the animals were infected with a patent infection when they started consuming heather) on the nematode fecal egg excretion and development of the adult worms. Moreover, one experimental group of animals that consumed heather in the curative experiment was administered polyethylene glycol (PEG), an inhibitor of condensed tannins, in order to elucidate whether the potential anthelmintic effect of heather was due or not to these compounds. In the second experiment we investigated the anthelmintic effect of heather consumption in goats experimentally infected with *T. circumcincta*. As in the previous experiments, the preventive and curative effect of heather consumption was studied.

Objective 3. Study of the *in vitro* effect of heather extracts on different parasitic stages of *Trichostrongylus colubriformis*, *Teladorsagia circumcincta* and *Haemonchus contortus*.

In our case, the aim of the *in vitro* experiments is putting a crude extract (containing tannins and other phenolic compounds) or a purified extract (which contains only condensed tannins) of heather into contact with the target parasite. The aim of these experiments is to avoid the influence of the host on the effect on the parasite, but always trying to replicate what happens *in vivo*. This procedure allows us to select the plant or plant material extracted from the plant species to be studied and the parasitic phase in a faster and cheaper way and under totally controlled conditions. In experiments that are developed in Chapter IV of this thesis, we used extracts of three species of heather (*Calluna vulgaris*, *Erica cinerea* and *Erica umbellata*) and a fourth extract composed by a mixture of the three species. Three studies were conducted to study the effect of extracts on different parasitic phases. To study the effect of extracts of heather on egg hatching, the EHA (Egg Hatch Assay) was used. To study the effect of heather extracts on third stage larvae exsheathment the LEIA assay (Larval exsheathment inhibition assay) was used. And finally, to investigate the effect of heather extracts on the adult worm mortality the AMIA assay (Adult Motility Inhibition Assay) was performed. Both egg hatching and exsheathment of infective larvae are key processes in the continuity of the life cycle of gastrointestinal nematodes therefore they have great relevance to the control of these parasites

CAPÍTULO II

Experimentos en condiciones naturales

Effects of stocking rate and heather supplementation on gastrointestinal nematode Infections and host performance in naturally infected Cashmere goats

Koldo Osoro,[1] Rafael Celaya,[2] Javier Moreno-Gonzalo,[3] Luis M.M. Ferreira,[4] Urcesino García,[5] Pilar Frutos,[6] Luis M. Ortega-Mora,[7] and Ignacio Ferre[7]

Authors are [1]Research Leader, [2]Research Associate, and [5]Experimental Farm Manager, Área de Sistemas de Producción Animal, Servicio Regional de Investigación y Desarrollo Agroalimentario, 33300 Villaviciosa, Asturias, Spain; [3]Graduate Student, and [7]Professors, Salud Veterinaria y Zoonosis, Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain; [4]Professor, Centro de Ciencia Animal e Veterinaria, Departamento de Zootecnia, Universidade de Trás-os-Montes e Alto Douro, PO Box 1013, 5000-911 Vila Real, Portugal; and [6]Research Associate, Estación Agrícola Experimental, Consejo Superior de Investigaciones Científicas, Finca Marzanas, 24346 León, Spain

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Abstract

The aim of this study, performed on 62 adult dry Cashmere goats grazing upland perennial ryegrass-white clover pastures and naturally infected with gastrointestinal nematodes, was to investigate the effects of stocking rate (SR: 24 vs. 38 goats/ha) and tannin-containing heather supplementation (H: *Calluna vulgaris* (L.) Hull, *Erica* spp. vs. non-supplementation) on parasite burden, fecal egg counts (FEC), and live weight (LW) changes. Goats were randomly assigned to four treatments in a 2 x 2 factorial arrangement and grazed continuously from May to October. Six goats per treatment were slaughtered at the end of the grazing period, with adult worms in the abomasum and small and large intestines of each animal recovered, counted, and identified. FEC was affected by SR ($P < 0.01$) but not by H. However, the SR x H interaction was significant ($P < 0.05$). FEC increased ($P < 0.001$) along the grazing season in all treatments, and the SR x Time interaction was significant ($P < 0.001$). In general, mean total worm counts in abomasum and small intestine tended to be higher under high SR, although the differences were only significant ($P < 0.01$) in *Trichostrongylus* spp counts. In goats managed under the high SR, the mean of total *Teladorsagia circumcincta* counts was lower ($P < 0.01$) in supplemented animals, but no differences were recorded for *Trichostrongylus* spp., *Chabertia ovina*, *Oesophagostomum columbianum* and *Trichuris ovis*. The goats gained more LW ($P < 0.001$) under low SR and when they were heather-supplemented. No significant SR x H interaction was found for LW change. In conclusion, high stocking rate increases the infectivity risk of pasture and the supplementation of grazing goats with heather contribute to improve animals' performance. Notwithstanding, the effect of heather availability on nematode FEC reduction could be highly dependent on the climatic conditions.

Resumen

Con el fin de estudiar el efecto de la carga ganadera (CG: 24 vs. 38 cabras/ha) y de la suplementación con brezo (SB: *Calluna vulgaris* (L.) Hull, *Erica* spp. vs. no brezo) en las parasitosis por nematodos gastrointestinales del ganado caprino y su repercusión en las variaciones de peso, se manejaron 62 cabras cachemir adultas sin cría en pastoreo continuo de mayo a octubre en pastos de raigrás-trébol. Las cabras se distribuyeron en cuatro grupos según un diseño factorial 2 x 2. Al finalizar el pastoreo se sacrificaron seis cabras por tratamiento para contar el número de vermes en abomaso e intestino delgado y grueso, e identificarlos. El recuento fecal de huevos de nematodos estaba afectado ($P < 0,01$) por CG, pero no por SB; sin embargo la interacción CG x SB resultó significativa ($P < 0.05$). La cantidad de huevos se incrementó ($P < 0,001$) durante la estación de pastoreo en todos los tratamientos y la interacción CG x Tiempo resultó significativa ($P < 0,001$). En general, el recuento de vermes en abomaso, intestino delgado y grueso tendió a ser mayor en los animales manejados en carga alta, aunque las diferencias fueron significativas ($P < 0,01$) solo para *Trichostrongylus* spp. En carga alta, la media total de *Teladorsagia circumcincta* fue menor ($P < 0,01$) en las cabras suplementadas, pero no se observaron diferencias para *Trichostrongylus* spp., *Chabertia ovina*, *Oesophagostomum columbianum* y *Trichuris ovis*. Las ganancias de peso fueron mayores ($P < 0,001$) en las cabras en carga baja y en las suplementadas, no observándose interacción CG x SB. En conclusión, en carga alta se observa un mayor riesgo de infección parasitaria, y la suplementación con brezo contribuye a mejorar las variaciones de peso de las cabras, aunque su efecto sobre los nematodos gastrointestinales podría depender de las condiciones climáticas.

Keywords: anthelmintic, grazing, live weight, parasite, tannin

1. Introduction

Conventional methods to control gastrointestinal nematode (GIN) infections of grazing ruminants have widely included the use of anthelmintic drugs. However, the resistance to all the major groups of broad-spectrum anthelmintics throughout the world (Jackson and Coop 2000) and the increasing demand by consumers for organic livestock products have prompted the investigation of alternative non-chemical control methods (Waller 2006). Stear et al. (2007)

divided the possible non-chemical methods in five categories: grazing management, biological control, nutrition, vaccination and genetic approaches. These authors indicated that no method can be recommended to the exclusion of all the others, and that there may be a role for a combination of methods.

Among grazing management methods, management decisions may modify the extent and severity of GIN infections (Morley and Donald 1980). Within these strategies, stocking rate, which affects sward height availability, is an intrinsic factor of the grazing systems that could be considered to be the main management factor affecting also biodiversity and systems sustainability (Milne and Osoro 1997). It is commonly assumed that increasing the stocking rate increases the level of parasitism in grazing animals. However, experimental evidence to support this view is rather limited in sheep, especially in temperate regions (Le Jambre 1984; Thamsborg et al. 1996), and is unknown in goats. The studies carried out in sheep (Thamsborg et al. 1996; Thamsborg and Hauge 2001) showed a very complex relationship between stocking rates and levels of infection by different parasites. Moreover, Osoro and Martínez (1995) observed that the percentage of improved pasture may affect parasite dynamics in goats managed on heathlands.

Within biological methods, a number of reports have been published in the last few years on the anthelmintic effects of tannin-rich plants on GIN infections in small ruminants (Hoste et al. 2006). In naturally infected goats, these studies showed a decrease in the establishment of third-stage nematode larvae or reductions in worm fertility and egg output when a moderate concentration of tannin-rich plants was consumed (Hoste et al. 2006; Shaik et al. 2006; Osoro et al. 2007b).

Surprisingly, there is a lack of research on the combination of different non-chemical methods, despite the idea that an integrated approach should achieve a better and more complete control of GIN infections (Barger 1999; Hoste et al. 2005, 2006). The objective of the present study was therefore to investigate the combination of two alternative methods: grazing management (stocking rate) and biological control (condensed tannins-heather supplementation) on GIN infections and animal performance in Cashmere goats grazing upland perennial ryegrass-white clover pastures. The study was conducted under practical conditions in a less-favored area where grazing animals are extensively raised and included in the natural ecosystem dynamics.

2. Materials and methods

Experimental site

The study was carried out in a mountainous area (1 000 m a.s.l.) in the northwest of Spain (lat 43º21'N, long 6º53'W, Sierra de San Isidro, Illano, Asturias), where shrubby heather-gorse vegetation is dominant. Four plots of 5 000 m^2 each were established in which the vegetation had been improved in 2001 by soil ploughing and dressing and sowing perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.).

Annual rainfall in the experimental year was 1 253 mm. During the grazing season, mean monthly rainfalls ranged from 11 mm in July to 166 mm in October. Mean average temperatures ranged from 11.3ºC in May to 16.9ºC in June.

Goats

A total of 62 adult non-lactating and non-pregnant Cashmere goats, reared outdoors under pasture conditions not excluding GIN infections, were used in this study. The mean live weight (LW) of the goats at the beginning of the experiment was 31.2 kg ± 0.88 SE. Three weeks before the experiment started all goats were dosed orally with ivermectin (Oramec™, Merial, Lyon, France). The efficacy of the anthelmintic treatment was assessed at 21 d post-treatment by means of individual examination of fecal egg counts (FEC) and inspection of clinical signs to confirm that animals did not show patent infection.

The experiment was carried out in accordance with Spanish Royal Decree 1201/2005 for the protection of animals used for experimental and other scientific purposes.

Experimental design

Sixty-two goats were balanced for LW and body condition and randomly assigned to one of four treatment groups in a 2 x 2 factorial arrangement. The main effects were two stocking rates (24 vs. 38 goats/ha) and two feeding treatments (supplementation with heather vs. non-supplementation). Supplemented groups were offered freshly cut heather *ad libitum* in a cage in the field twice a week in the morning, whereas the others received no supplement. All goats grazed continuously throughout the whole experiment (there was not night confinement). The trial extended from 6 May to 21 October.

Sampling procedures

Pasture. In order to control grass availability, sward surface height was measured weekly using a HFRO swardstick (Barthram 1986), with 100 measurements taken at random in each plot. The botanical composition of the pasture was assessed during the summer (21 July) using a point quadrant (Grant 1981) and recording 100 vertical hits at random in each plot.

Ash and nitrogen (N) of pasture sampled in June, August, and October were analyzed following the procedures of the Association of Official Analytical Chemists (AOAC 2006). Crude protein (CP) was calculated as N x 6.25. Neutral and acid detergent fibre (NDF and ADF) and acid detergent lignin (ADL) were analyzed by the method of Goering and van Soest (1970) and van Soest et al. (1991).

Heather. The species composition of the heather offered to the goats was assessed in July by recording 1 000 random contacts with the HFRO swardstick throughout the area where the heather was cut. Samples of the green shoots (less than 3 mm in diameter) of the heather offered to the goats were collected in June, August, and September for chemical composition. The nutritive quality (ash, CP, NDF, ADF, and ADL) of the green shoots was analyzed following the same procedures mentioned for the pasture. Heather offered to the supplemented goats was also analyzed for total tannins using the Folin-Ciocalteu assay in combination with polyvinyl-polypyrrolidone, as described by Makkar et al. (1993). Total tannin content was expressed as tannic acid equivalents.

Diet selection. The percentage of heather in the diet of each individual goat was estimated on 19 August using the *n*-alkane method (Mayes et al. 1986), as modified by Oliván and Osoro (1999). Samples of pasture, heather, and feces from individual animals were collected, and calculations performed using a least squares optimization procedure (Dove and Moore 1995), which minimizes the discrepancies between the actual concentration of alkanes in feces (adjusted for incomplete fecal recoveries, using the recovery values obtained in previous studies by Ferreira et al. 2005) and the estimated proportion in the diet.

Parasitological procedures. Throughout the six-month trial, fecal samples were collected monthly from individual animals for fecal egg count (FEC) analysis. Feces were collected directly from the rectum, with ova counted using a modified McMaster procedure (MAFF 1978) with sodium chloride as the flotation medium, in which 1 egg counted is equivalent to 15 eggs per gram (epg) fresh feces. Fecal cultures were prepared monthly for each group by pooling samples from individual goats as described by MAFF (1978) to allow identification of third-stage nematode larvae genera according to van Wyk et al. (2004). In each sample 200 third-stage larvae were identified.

At the end of the trial, six goats of each treatment were slaughtered and adult worms were recovered from the abomasum and intestines. After slaughter, the abomasum and small and large intestines of each goat were ligated and opened, and the contents washed on a mesh screen (60 µm) with tap water. For abomasum and small intestine, the contents were brought up to 500 mL with tap water and thoroughly mixed, and then one 10% aliquot (50 mL) was moved into 100-mL storage containers. Approximately 100 mL of formalin 100% was added to each aliquot as a preservative. For both abomasal and small intestinal samples, the worms in the aliquot were washed on a mesh screen (60 µm) with tap water, the formalin discarded, and the nematodes recovered into a 50-mL centrifuge tube. All large intestine contents were examined for GIN recovery. Afterwards, the nematodes were counted and identified according to species and sex using a phase contrast microscope.

In order to assess the nematode development, the lengths of 20 male and 20 female worms of each species were measured using a microscope at 10x magnification. Fertility per female worm of each species was determined by direct counting of the eggs *in utero* after clearing of each female worm with lactophenol for *Teladorsagia circumcincta* and *Trichostrongylus* spp. according to the method described by Kloosterman et al. (1978) for *Haemonchus contortus*. Counts were performed on at least 20 female worms per goat.

Live weight and body condition score. All animals were weighed at the beginning and the end of the experimental grazing season and also at monthly intervals. At the same time, the body condition score (BCS) was assessed on a scale of 1 to 5 (1 = emaciated, 5 = fat; Russel 1990). To study LW changes and the possible relationship with FEC and heather percentage in the diet, the grazing season was divided into two periods: period 1 from the beginning to 19 August (high sward availability), and period 2 from 19 August until the end of the grazing season on 21 October (low sward availability).

Statistical analyses

All analyses were performed using SAS System software (SAS Institute 1999). FEC data from individual animals were analyzed using a Mixed Model procedure (PROC MIXED) for repeated measures (Littell et al. 1998; Wang and Goonewardene 2004), including in the model the fixed effects of stocking rate (SR), heather supplementation (H), sampling time (T, with six repeated measures), and the interactions SR x H, SR x T, H x T and SR x H x T, with the first measure in May as covariate.

Sward height data (plot means) were subjected to PROC MIXED, with 12 repeated measures in time (T) across the grazing season, examining the effects of stocking rate and heather supplementation independently, and the interactions SR x T or H x T, respectively.

Data on goat LW and BCS changes in periods 1 and 2, and GIN measurements (parasite burden, sex ratio, fecundity *in utero*, body length) on euthanized goats at the end of the trial were analyzed with a General Linear Model procedure (PROC GLM) for the effects of the two main factors, SR and H, and their interaction. Pasture botanical composition in July (hit percentages from each plot) was analyzed by 1-way ANOVA to test for SR or H effects independently. Diet composition of supplemented goats in August was analyzed by 1-way ANOVA for the SR effect.

FEC data and worm counts were log transformed ($\log_{10} x + 1$) to normalize their distribution.

Correlation coefficients between heather percentage in the diet, FEC, and LW changes were analyzed using Spearman rank-order correlation. The mortality rate of goats in the different treatments was analyzed by chi-square test.

3. Results

Pasture

Sward height. Sward height achieved maximum values in June (19.7 cm and 15.5 cm at low and high stocking rates, respectively) and decreased until August, remaining constant during period 2 (Figure 1). Significant differences (P < 0.01) in the mean sward height across the grazing season were observed between low and high stocking rates (11.2 cm and 9.2 cm, respectively). The mean sward heights during period 1 were 13.4 cm under the low stocking rate and 11.2 cm under the high stocking rate. During period 2, the means were 7.7 cm and 6.2 cm under the low and high stocking rate, respectively. No significant differences in sward height were observed as a consequence of heather supplementation.

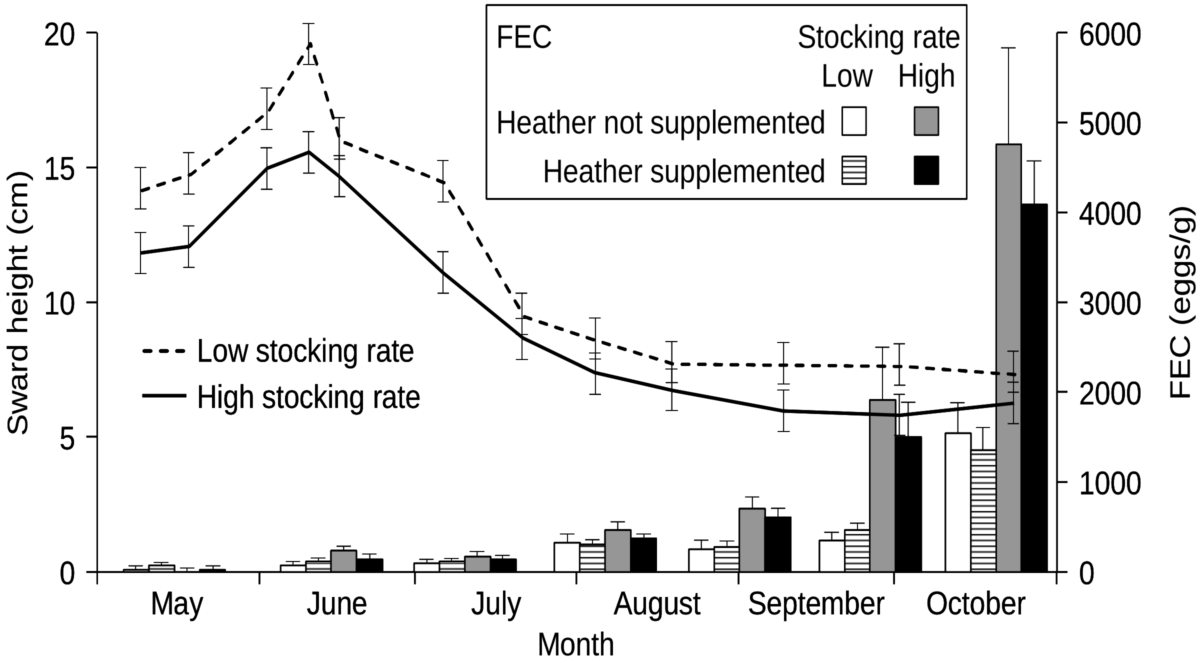

Figure 1. Sward height (means ± SEM) during the experimental grazing period and fecal egg counts (FEC, means ± SE) in goats grazing perennial ryegrass-white clover pastures under two stocking rates (Low: 24 goats/ha, High: 38 goats/ha) and supplemented or not with heather.

Table 1. Chemical composition (mean ± S.E.) of pasture and heather across the grazing period.

	Pasture			Heather		
(g·kg^{-1} DM)[1]	June	August	October	June	August	September
OM	932 ± 3.3	957 ± 15.0	945 ± 9.1	977 ± 0.3	968 ± 11.4	977 ± 7.9
NDF	595 ± 51.2	690 ± 48.5	709 ± 27.0	565 ± 28.9	573 ± 5.3	686 ± 14.7
ADF	288 ± 34.6	389 ± 52.6	388 ± 51.4	508 ± 10.8	510 ± 11.5	506 ± 9.8
ADL	35 ± 8.4	86 ± 22.0	61 ± 13.5	347 ± 7.5	333 ± 15.7	352 ± 7.9
CP	161 ± 28.1	118 ± 31.7	123 ± 20.1	71 ± 28.8	71 ± 2.9	57 ± 5.7
Tannins[2]				97 ± 5.9	61 ± 4.4	79 ± 4.9

[1]DM indicates dry matter; OM: organic matter; NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL: acid detergent lignin; CP: crude protein. [2]Expressed in tannic acid equivalents.

Botanical composition. No significant differences were found in the sward composition, studied in July, between high and low stocking rates or between supplemented and non-supplemented treatment plots. Green ryegrass accounted for a mean percentage of 27% ± 1.0 SE, while the percentage of the native grass *Agrostis capillaris* L. was 26% ± 3.9. White clover accounted for a mean of 4% ± 1.2. The percentage of dead matter was high (43% ± 3.1) as a consequence of the summer drought. The percentage of grass flower stems was also high (21% ± 2.5) compared to the percentage of grass green foliage (32% ± 3.8).

Chemical composition. As shown in Table 1, pasture chemical composition changed from the beginning of the grazing period (June) to summer-autumn. The protein content decreased from 161 $g \cdot kg^{-1}$ dry matter (DM) to 118 $g \cdot kg^{-1}$ DM, and the cell wall components (NDF and ADF) increased from 595 $g \cdot kg^{-1}$ DM to 709 $g \cdot kg^{-1}$ DM and from 288 $g \cdot kg^{-1}$ DM to 389 $g \cdot kg^{-1}$ DM, respectively.

Heather

Botanical composition. Among the offered heather species, *C. vulgaris* attained the highest percentage (57%), while *Erica umbellata* L., *Erica cinerea* L., *Erica tetralix* L. and *Daboecia cantabrica* (Hudson) C. Koch accounted for 26%, 13%, 3%, and 1%, respectively.

Chemical composition. Heather protein content during the experimental season ranged between 57 $g \cdot kg^{-1}$ DM and 71 $g \cdot kg^{-1}$ DM (Table 1). Cell wall components did not change significantly along the experimental season, with the exception of NDF, which increased from August (573 $g \cdot kg^{-1}$ DM) to September (686 $g \cdot kg^{-1}$ DM). Tannin content ranged from 97 $g \cdot kg^{-1}$ DM in June to 61 $g \cdot kg^{-1}$ DM in August. (Table 1).

Diet composition

The proportion of heather in the diet of supplemented goats was not affected by the stocking rate (0.201 ± 0.023 and 0.198 ± 0.018 for low and high stocking rate, respectively).

Fecal egg counts and coprocultures

The FECs increased significantly ($P < 0.001$) across the grazing season and a significant ($P < 0.01$) interaction was found between stocking rate and time (Figure 1). In September and October samplings, the animals from plots under the high stocking rate showed significant ($P < 0.01$) higher mean FECs than those on the lower stocking rate. No differences in FEC were detected between goats supplemented with heather and non-supplemented. However, a significant ($P < 0.05$) interaction was found between stocking rate and heather supplementation for the overall mean values across the grazing season.

Nematode genera, identified based on third-stage larvae obtained from coprocultures, were *Teladorsagia*, *Trichostrongylus* and *Chabertia* (Figure 2). *Teladorsagia* was the most frequently identified in June and July (60-93%) in the four plots, decreasing sharply afterwards. In contrast, *Trichostrongylus* was predominant from August to October (33-100%). *Chabertia* was detected in all plots but at a lower percentage (0-31%), except in August sampling on goats not supplemented with heather under the higher stocking rate (52%). The female worm fertility (estimated by the numbers of eggs *in utero*, Table 2) and development (estimated by the total length, Table 3) were significantly ($P < 0.05$) lower for *T. axei* and *Trichostrongylus* spp. in nematodes from goats under the higher stocking rate. The fertility of *Teladorsagia circumcincta* tended to be higher in worms from goats under the high stocking rate, particularly in those from supplemented goats. In general, the number and length of male worms tended to be lower in goats under the higher stocking rate and supplemented with heather.

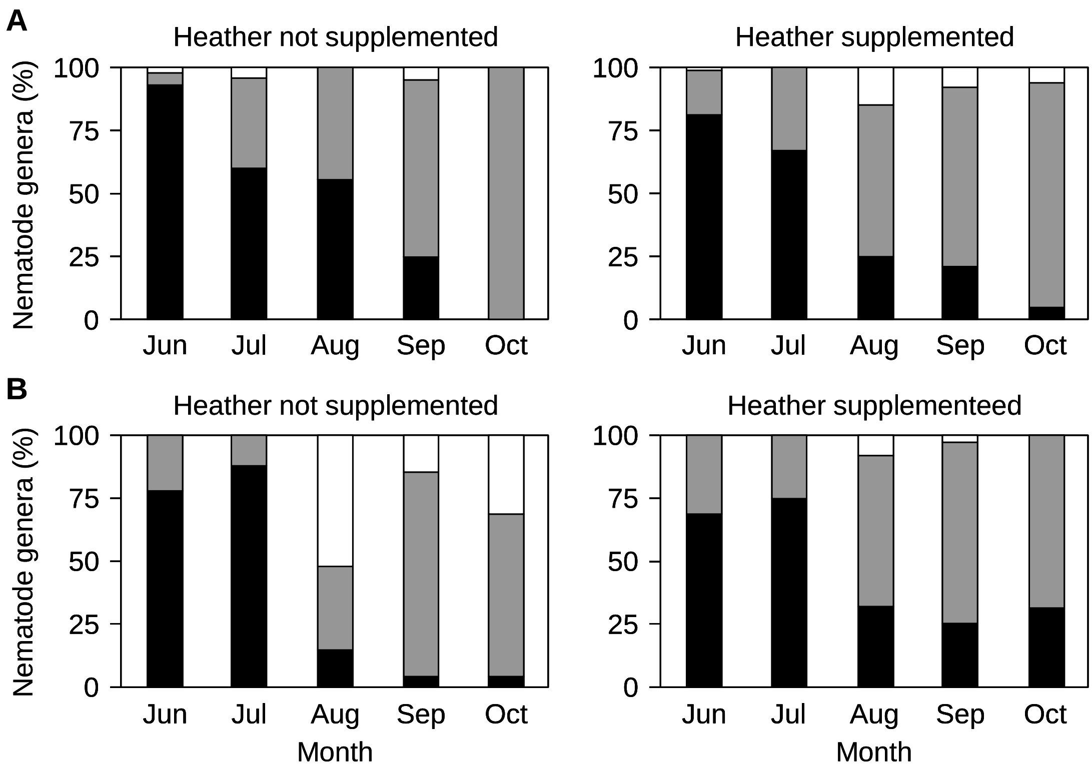

Figure 2. Nematode genera identified on third-stage larvae obtained in coprocultures from goats grazing perennial ryegrass-white clover pastures under two stocking rates and supplemented or not with heather. **A**, Low stocking rate (24 goats/ha). **B**, High stocking rate (38 goats/ha). Black: *Teladorsagia*; grey: *Trichostrongylus*; white: *Chabertia*.

Mortality of goats

A total of nine goats died between September and October, eight animals corresponding to plots with 38 goats/ha (three supplemented and five non-supplemented with heather) and one supplemented goat from the plot with 24 goats/ha. Although the mortality rate tended to be higher (P = 0.057) in goats under the high stocking rate, no significant differences were associated with heather supplementation (P > 0.10).

Live weight and body condition changes

The effect of stocking rate on LW changes was only significant (P < 0.001) for the second period of the grazing season (Table 4). In the first period, goats under the low stocking rate gained only 7 $g \cdot d^{-1}$ more than goats under the high stocking rate, whereas they lost 46 $g \cdot d^{-1}$ less in the second period. On the other hand, heather supplementation significantly affected LW changes, both in the first (P < 0.05) and second (P < 0.01) grazing periods. Goats receiving heather supplementation gained 17 $g \cdot d^{-1}$ more in the first period and lost 21 $g \cdot d^{-1}$ less in the second period than non-supplemented goats.

LW changes for the overall grazing season were significantly (P < 0.001) affected by the stocking rate (gains of 30 *vs*. 9 $g \cdot d^{-1}$ for low and high, respectively) and heather supplementation (gains of 28 *vs*. 11 $g \cdot d^{-1}$ for supplemented and non-supplemented goats). The interaction of stocking rate x heather supplementation was not significant, although a tendency (P < 0.10) was observed in the second period, with non-supplemented goats under the high stocking rate showing the greatest weight loss (-117 $g \cdot d^{-1}$).

Table 2. Mean worm burden (counts/goat), sex ratio (male/female) and fecundity *in utero* (eggs/female) of abomasal and intestinal nematodes in goats under different stocking rates and supplemented or non-supplemented with heather.

	Low (24 goats/ha)		High (38 goats/ha)		
	No	Yes	No	Yes	SEM
Abomasum (total)	4 477 ab	3 476 b	6 998 a	3 693 b	1 003
Teladorsagia circumcincta					
Worm burden	4 129 ab	3 172 b	6 209 a	3 099 b	922
Sex ratio	0.76 a	0.78 a	0.66 b	0.68 b	0.044
Fecundity in utero	6.6 b	5.6 b	7.7 ab	9.5 a	0.78
Trichostrongylus axei					
Worm burden	348 b	304 b	789 a	594 a	173
Sex ratio	0.10 b	0.76 a	0.14 b	0.92 a	0.094
Fecundity in utero	15.4 a	13.1 a	10.0 b	9.4 b	1.08
Small intestine (total)	7 671 b	7 077 b	24 733 a	24 592 a	3 538
Trichostrongylus spp.					
Worm burden	7 671 b	7 077 b	24 733 a	24 592 a	3 538
Sex ratio	0.91 a	0.90 a	0.85 ab	0.80 b	0.036
Fecundity in utero	18.8 a	14.8 a	11.2 b	9.6 b	1.30
Large intestine (total)	24.8	25.6	26.6	28.5	8.22
Chabertia ovina					
Worm burden	0.4 b	0.8 b	17.6 a	6.5 b	2.07
Oesophagostomum columbianum					
Worm burden	10.8	7.0	4.6	6.8	3.06
Trichuris ovis					
Worm burden	13.6	17.8	4.1	15.2	7.83

Means followed by different letters in the same row are significantly different ($P < 0.05$).

Table 3. Mean length (mm) of abomasal and intestinal nematodes in goats under different stocking rates and supplemented or non-supplemented with heather.

	Low (24 goats/ha)		High (38 goats/ha)		
	No	Yes	No	Yes	SEM
Teladorsagia circumcincta		43			
Female	7.21 b	7.63 a	6.98 b	7.66 a	0.182
Male	5.88 a	5.90 a	5.61 b	5.40 b	0.128
Trichostrongylus axei					
Female	5.88 a	5.98 a	5.28 b	5.11 b	0.195
Male	4.66 a	4.74 a	4.20 b	4.13 b	0.153
Trichostrongylus spp.					
Female	6.50 a	6.43 a	5.92 ab	5.55 b	0.189
Male	5.14	4.95	4.64	4.70	0.183

Means followed by different letters in the same row are significantly different ($P < 0.05$).

Body condition was affected by stocking rate in a similar way to LW changes ($P < 0.001$). However, the effect of heather supplementation was not statistically significant in any period (Table 4).

Correlations

The study of the relationships between the heather consumption by goats and their parasite burden and performance showed a significant positive correlation ($r = 0.39$, $P < 0.01$) between LW changes during the grazing season and the percentage of heather in the diet, which ranged from 10% to 45% in the supplemented goats. The correlations were significant for both low ($r = 0.56$, $P < 0.01$) and high ($r = 0.47$, $P < 0.01$) stocking rates. On the contrary, significant negative correlations ($r = -0.59$, $P < 0.001$) were found between LW changes and FEC in October. No significant relationship between the percentage of heather in the diet and FEC was observed.

4. Discussion

Although the results reported in the literature are not consistent, a significant effect of the stocking rate on GIN infection was observed in this study. In the second half of the grazing season (period 2), FEC increased considerably under the high stocking rate (from 360-470 epg in August to 4 100-4800 epg in October) while the increase was more limited at the low stocking rate (from 300 epg to 1500 epg). This effect of stocking rate on FEC was in accordance with previous results obtained in Angora goats naturally infected with *Teladorsagia circumcincta* and *Haemonchus contortus* (Le Jambre 1984) and in grazing sheep (Thamsborg et al. 1996). Hansen et al. (1989) also found in beef heifers that the lowest stocking rate groups continuously had the lowest GIN egg output and serum pepsinogen levels. Animals at higher stocking rates are forced to graze closer to the manure, and they inevitably ingest a larger number of infective nematode larvae. In general, a higher stocking rate means higher GIN egg output and dispersion and therefore higher pasture contamination and infection risk. In addition, mountain pastures have a higher density than those in lowlands, and the development and persistence of third-stage trichostrongylid larvae is higher in denser pastures (Moss and Bray 2006). A high stocking rate also means a shorter palatable sward height, less options to select grazing areas when rejecting those contaminated by feces, lower intake, and even negative nutritive balances (Morley and Donald 1980).

Contrary to previous studies in the same experimental farm (Osoro et al. 2007a, 2007b), in the present trial no significant differences in FEC were detected between goats supplemented with tannin-containing heather and those non-supplemented. The lack of significant differences in the current study could be due to the lower nematode burden when compared to previous grazing seasons, which was probably caused by an anomalous dry year. In previous studies (Osoro et al. 2007b), the mean FEC at the end of the grazing season in the non-supplemented group, which was managed under a lower stocking rate, was 8600 epg compared to 1500 epg and 4500 epg observed in the present trial under low and high stocking rates, respectively, in non-supplemented goats. Climatic conditions are known to affect GIN larvae survival and migration in the environment and subsequently the intensity of infection. The dispersal of larvae from feces is largely dependent on sufficient water from dew or rainfall to allow migrating (Stromberg 1997).

The FEC kinetics over the grazing season and GIN genera identified in coprocultures confirmed previous epidemiological data obtained in the same goat research farm (Osoro et al. 2007b).

Our results showed a significant effect of stocking rate on *Trichostrongylus* spp. but not on *Teladorsagia circumcincta* worm burdens. These results are in agreement with those reported by Thamsborg and Hauge (2001), who observed higher FEC and worm burdens in grazing weaned lambs naturally infected with *T. circumcincta* and *Trichostrongylus vitrinus* at a high stocking rate when compared with animals at low or medium stocking rates. In the current work, heather supplementation reduced *T. circumcincta* burden, but this effect was only significant in goats under the high stocking rate. It is also noteworthy that fecundity *in utero* and length of *T. circumcincta* females in

Table 4. Effects of stocking rate and heather supplementation on goat live weight (LW) and body condition score (BCS) changes during the grazing season.

Stocking rate (SR)	Low (24 goats/ha)		High (38 goats/ha)			Significance		
Heather supplementation (H)	Yes	No	Yes	No	SEM	SR	H	SR x H
Initial LW (kg)	24.6	25.7	22.1	22.9	0.53	*	NS	NS
Initial BCS (scale 1 to 5)	2.8	2.7	2.7	2.6	0.03	NS	NS	NS
LW change ($g \cdot d^{-1}$)								
Period 1[1]	95	68	78	72	3.3	NS	*	NS
Period 2[2]	-51	-61	-86	-117	4.6	***	**	NS
Overall[3]	40	20	15	2	2.8	***	***	NS
BCS change (scale units)								
Period 1[1]	0.3	0.3	0.2	0.3	0.03	NS	NS	NS
Period 2[2]	0.1	0.0	-0.5	-0.5	0.38	***	NS	NS
Overall[3]	0.4	0.3	-0.3	-0.2	0.45	***	NS	NS

[1]From 6 May to 19 August. [2]From 19 August to 21 October. [3]From 6 May to 21 October. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS indicates not significant ($P > 0.05$)

supplemented goats were higher than those observed in non-supplemented animals, probably indicating a long term established population. Thus, heather could affect the development of the incoming third-stage *T. circumcincta* larvae. A reduction of 70% in worm numbers was observed by Paolini et al. (2003) in goats receiving condensed tannins before being infected with third-stage larvae of *T. circumcincta*.

In this trial, heather supplementation had a positive effect on LW changes and reduced GIN effects when animals were managed under the high stocking rate. Previous studies have reported the effect of protein on host resilience to GIN infection (van Houtert and Sykes 1996; Chartier et al. 2000). However, contrarily to other protein-rich tannin-containing legumes, such as *Lotus corniculatus* L., used in other studies (Marley et al. 2003, Athanasiadou et al. 2006), heather has a low protein content (approx. 70 g CP·kg DM^{-1}), and therefore the positive responses to its supplementation cannot be attributed to the effect of a dietary protein increase. Nevertheless, heather supplementation could have a positive influence on other aspects of protein metabolism such as reducing ruminal protein degradation through the action of tannins (Frutos et al. 2000).

The positive effect of supplementation of heather and forage containing condensed tannins on animals' performance was previously observed in grazing Cashmere (Osoro et al. 2007a, 2007b) and Angora goats (Min et al. 2005). In the current study, goats under the high stocking rate had significantly worse live weight changes in the second half of the grazing season when the available mean sward height was below 8 cm. These results are consistent with the linear relationship observed between green sward height and LW changes in Cashmere goats grazing pastures with sward height between 3 cm and 11 cm (Merchant and Riach 1994; Osoro et al. 2000), or between 6 cm and 11 cm in feral x Angora goats (Radcliffe et al. 1991). A restriction of goats' performance in pastures with sward height lower than 7 cm was reported by McCall and Lambert (1987). The absence of significant differences in LW changes during the first period would be a consequence of the high sward height (13.1 cm ± 1.08) not limiting herbage intake. The higher LW gains (68-95 $g \cdot d^{-1}$) observed in this study during the first period in comparison with a previous work carried out in the same farm (Osoro et al. 2007b), where goats had higher sward height available (19.5 cm ± 3.10) but lower LW gains (17-50 $g \cdot d^{-1}$), is an indicator of the effect of pasture quality and climatic conditions on LW changes. It is known that, according to pasture dynamics, there is an increase of pasture senescence associated with the increase in sward height

(Bircham and Hodgson 1983; Hodgson 1985), with goats showing preference for green pastures (Merchant and Riach 1994).

The interaction between nutrition, GIN infection, and LW changes has been well discussed both in sheep (Houdijk and Athanasiadou 2003) and goats (Hoste et al. 2005). It has been suggested that the severity of disease and the loss of production depend upon the intensity of infection, the host immunity, and the host´s relative nutritional status (Coop and Kyriazakis 2001).

The positive correlation coefficient between the heather percentage in the diet and the LW changes during the experimental grazing season in both stocking rates would be in agreement with our previous results (r = 0.54; Osoro et al. 2007b), and would point again to the beneficial effects of heather availability on goats' performance.

Implications

The high stocking rate increased the infection risk at pasture, and the supplementation of grazing goats with tannin-containing heather improved the animals' performance, but was not able to reduce the FEC under the dry climatic conditions of the study year. Practical application of this knowledge in temperate areas would support the management of plots integrating productive improved pastures with high nutritive value (ryegrass-white clover) with natural vegetation communities including tannin-containing plants, such as heather species, as previously suggested (Osoro et al. 1999; Kahiya et al. 2003). Improved pastures would be used to improve protein and energy intake, and natural vegetation for fibre, minerals, and secondary compounds (condensed tannins) that contribute to improve animal welfare and performance to achieve sustainable goat production systems.

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References

Association of Official Analytical Chemists. 2006. Official methods of analysis, 18th edition (1st revision). Gaithersburg, MD, USA: AOAC. 2200 p.

Athanasiadou, S., I. Kyriazakis, and F. Jackson. 2006. Can plant secondary metabolites have a role in controlling gastrointestinal nematode parasitism in small ruminants? *In*: C. A. Sandoval-Castro, F. D. De B. Hovell, J. F. J. Torres-Acosta and A. Ayala-Burgos [EDS.]. Herbivores: the assessment of intake, digestibility and the roles of secondary compounds. Nottingham, United Kingdom: British Society of Animal Science. p. 197-207.

Barger, I. A., 1999. The role of epidemiological knowledge and grazing management for helminth control in small ruminants. *International Journal of Parasitology* 29:41-47.

Barthram, G. T. 1986. Experimental techniques: the HFRO swardstick. *In*: The Hill Farming Research Organisation Biennial Report, 1984–85. Bush Estate, Penicuik, Midlothian, Scotland, United Kingdom: HFRO. p. 29-30.

Bircham, J. S., and J. Hodgson. 1983. The influence of sward condition on rates of herbage growth and senescence in mixed swards under continuous stocking management. *Grass and Forage Science* 38:323-331.

Chartier, C., E. Etter, H. Hoste, I. Pors, M.-P. Mallereau, C. Broqua, S. Mallet, C. Koch, and A. Massé. 2000. Effects of the initial level of milk production and of the dietary protein intake on the course of natural nematode infection in dairy goats. *Veterinary Parasitology* 92:1-13.

Coop, R. L., and I. Kyriazakis. 2001. Influence of host nutrition on the development and consequences of nematode parasitism in ruminants. *Trends in Parasitology* 17:325-330.

Dove, H., and A. D. Moore. 1995. Using a least-squares optimisation procedure to estimate diet composition based on the alkanes of plant cuticular wax. *Australian Journal of Agricultural Research* 46:1535-1544.

Ferreira, L. M. M., M. Oliván, U. García, M. A. M. Rodrigues, and K. Osoro. 2005. Validation of the alkane technique to estimate diet selection of goats grazing heather-gorse vegetation communities. *Journal of the Science of Food and Agriculture* 85:1636-1646.

Frutos, P., G. Hervás, F. J. Giráldez, M. Fernández, and A. R. Mantecón. 2000. Digestive utilisation of quebracho treated soya bean meals in sheep. *Journal of Agricultural Science* 134:101-108.

Goering, M. K., and P. J. van Soest. 1970. Forage fiber analysis (apparatus, reagents, procedures and some applications). Washington D.C., USA: US Department of Agriculture, Agricultural Research Service, Agricultural Handbook No.

379. 19 p.

Grant, S. A. 1981. Sward components. *In*: J. Hodgson, R. D. Baker, A. Davies, A. S. Laidlaw and J. D. Leaver [EDS.]. Sward measurement handbook. Hurley, Maidenhead, Berkshire, United Kingdom: British Grassland Society. p. 71-92.

Hansen, J. W., A. M. Zajac, D. E. Eversole, and H. J. Gerken. 1989. The effect of stocking rate and parasite control on the performance of replacement beef heifers on pasture. *Veterinary Parasitology* 34:103-113.

Hodgson, J. 1985. The control of herbage intake in the grazing ruminant. *Proceedings of the Nutrition Society* 44:339-346.

Hoste, H., J. F. Torres-Acosta, V. Paolini, A. Aguilar-Caballero, A. Etter, Y. Lefrileux, C. Chartier, and C. Broqua. 2005. Interactions between nutrition and gastrointestinal infections with parasitic nematodes in goats. *Small Ruminant Research* 60:141-151.

Hoste, H., F. Jackson, S. Athanasiadou, S. M. Thamsborg, and S. O. Hoskin. 2006. The effects of tannin-rich plants on parasitic nematodes in ruminants. *Trends in Parasitology* 22:253-261.

Houdijk, J. G. M., and S. Athanasiadou. 2003. Direct and indirect effects of host nutrition on ruminant gastrointestinal nematodes. *In*: L. 't Mannetje, L. Ramírez-Avilés, C. Sandoval-Castro and J. C. Ku-Vera [EDS.]. Matching herbivore nutrition to ecosystems biodiversity. VI International Symposium on the Nutrition of Herbivores. Mérida, Yucatán, México: Universidad Autónoma de Yucatán. p. 213-236.

Jackson, F., and R. L. Coop. 2000. The development of anthelmintic resistance in sheep nematodes. *Parasitology* 120:95-107.

Kahiya, C., S. Mukaratirwa, and S. M. Thamsborg. 2003. Effects of *Acacia nilotica* and *Acacia karoo* diets on *Haemonchus contortus* infection in goats. *Veterinary Parasitology* 115:265-274.

Kloosterman, A., G. A. A. Albers, and H. Van Den Brink. 1978. Genetic variations among calves in resistance to nematode parasites. *Veterinary Parasitology* 4:353-368.

Le Jambre, L. F. 1984. Stocking rate effects on the worm burdens of Angora goats and Merino sheep. *Australian Veterinary Journal* 61:280-282.

Littell, R. C., P. R. Henry, and C. B. Ammerman. 1998. Statistical analysis of repeated measures data using SAS procedures. *Journal of Animal Science* 76:1216-1231.

[MAFF] Ministry of Agriculture, Fisheries and Food. 1978. Manual of veterinary parasitological techniques. London, United Kingdom: MAFF, Technical Bulletin No. 18. 129 p.

Makkar, H. P. S., M. Blummel, N. K. Borowy, and K. Becker. 1993. Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. *Journal of the Science of Food and Agriculture* 61:161-165.

Marley, C. L., R. Cook, R. Keatinge, J. Barret, and N. H. Lampkin. 2003. The effect of birdsfoot trefoil (*Lotus corniculatus*) and chicory (*Cichorium intybus*) on parasite intensities and performance of lambs naturally infected with helminth parasites. *Veterinary Parasitology* 112:147-155.

Mayes, R. W., C. S. Lamb, and P. M. Colgrove. 1986. The use of dosed and herbage *n*-alkanes as markers for the determination of herbage intake. *Journal of Agricultural Science* 107:161-170.

McCall, D. G., and M. G. Lambert. 1987. Pasture feeding of goats. *In*: A. M. Nicol [ED.]. Livestock feeding on pasture. Hamilton, New Zealand: New Zealand Society of Animal Production, Occasional Publication No. 10. p. 105-109.

Merchant, M., and D. J. Riach. 1994. The intake and performance of cashmere goats grazing sown swards. *Grass and Forage Science* 49:429-437.

Milne, J., and K. Osoro. 1997. The role of livestock in habitat management. *In*: J.P. Laker and J. A. Milne [EDS.]. Livestock systems in European rural development. Proceedings of the 1st Conference of the LSIRD network, Nafplio, Greece. Craigiebuckler, Aberdeen, Scotland, United Kingdom: Macaulay Land Use Research Institute. p. 75-80.

Min, B. R., S. P. Hart, D. Miller, G. M. Tomita, E. Loetz, and T. Sahlu. 2005. The effect of grazing forage containing condensed tannins on gastro-intestinal parasite infection and milk composition in Angora does. *Veterinary Parasitology* 130:105-113.

Morley, F. H. W., and A. D. Donald. 1980. Farm management and systems of helminth control. *Veterinary Parasitology* 6:105-134.

Moss, R. A., and A. R. Bray. 2006. Effect of sward density and size of faecal deposit on the development and persistence of third-stage Trichostrongylid larvae of sheep. *New Zealand Journal of Agricultural Research* 49:475-481.

Oliván, M., and K. Osoro. 1999. Effect of temperature on alkane extraction from faeces and herbage. *Journal of Agricultural Science* 132:305-312.

Osoro, K., and A. Martínez. 1995. Grazing behaviour and performance of goats and sheep on natural and improved vegetation. *In*: J. P. Laker, and A. J. F. Russel [EDS.]. The nutrition and grazing ecology of speciality fibre producing animals. Craigiebuckler, Aberdeen, Scotland, United Kingdom: Macaulay Land Use Research Institute, European Fine Fibre Network Occasional Publication No. 3. p. 109-125.

Osoro, K., J. M. Vassallo, R. Celaya, and A. Martínez. 1999. Livestock production systems and the vegetation dynamics of Less Favoured Areas (LFAs): developing viable systems to manage semi-natural vegetation in temperate LFAs in Spain. *In*: J. P. Laker and J. A. Milne [EDS.]. Livestock production in the European Less Favoured Areas: Meeting future economic, environmental, and policy objectives through integrated research. Proceedings of the 2nd conference of the LSIRD network, Bray, Dublin, Ireland. Craigiebuckler, Aberdeen, Scotland, United Kingdom: Macaulay Land Use Research Institute. p. 133-143.

Osoro, K., R. Celaya, and A. Martínez. 2000. The effect of grazing management of sheep and goats on animal performance and vegetation dynamics in partially improved heath-gorse vegetation. *In*: A. J. Rook and P. D. Penning [EDS.]. Grazing management. Reading, United Kingdom: British Grassland Society, Occasional Symposium No. 34. p. 135-140.

Osoro, K., A. Benito-Peña, P. Frutos, U. García, L. M. Ortega-Mora, R. Celaya, and I. Ferre. 2007a. The effect of heather supplementation on gastrointestinal nematode infections and performance in Cashmere and local Celtiberic goats on pasture. *Small Ruminant Research* 67:184-191.

Osoro, K., A. Mateos-Sanz, P. Frutos, U. García, L. M. Ortega-Mora, L. M. M. Ferreira, R. Celaya, and I. Ferre. 2007b. Anthelmintic and nutritional effects of heather supplementation on Cashmere goats grazing perennial ryegrass-white clover pastures. *Journal of Animal Science* 85:861-870.

Paolini, V., A. Frayssines, F. De la Farge, P. Dorchies, and H. Hoste. 2003. Effects of condensed tannins on established populations and on incoming larvae of *Trichostrongylus colubriformis* and *Teladorsagia circumcincta* in goats. *Veterinary Research* 34:331-339.

Radcliffe, J. E., R. J. Townsend, and D. B. Baird. 1991. Mixed and separate grazing of sheep and goats at two stocking rates. *New Zealand Journal of Agricultural Research* 34:167-176.

Russel, A. J. F. 1990. Body condition scoring of goats. Scottish Cashmere Producers Association Newsletter 10:3.

SAS Institute. 1999. SAS/STAT user's guide, V8. Cary, NC, USA: SAS Institute, Inc. 3884 p.

Shaik, S. A., T. H. Terrill, J. E. Miller, B. Kouakou, G. Kannan, R. M. Kaplan, J. M. Burke, and J. A. Mosjidis. 2006. Sericea lespedeza hay as a natural deworming agent against gastrointestinal nematode infection in goats. *Veterinary Parasitology* 9:150-157.

Stear, M. J., M. Doligaska, and K. Donskow-Schmelter. 2007. Alternatives to anthelmintics for the control of nematodes in livestock. *Parasitology* 134:139-151.

Stromberg, B. E. 1997. Environmental factors influencing transmission. *Veterinary Parasitology* 72:247-264.

Thamsborg, S. M., and E. M. Hauge. 2001. Osteopenia and reduced serum alkaline phosphatase activity in grazing lambs naturally infected with gastrointestinal nematodes. *Journal of Comparative Pathology* 125:192-203.

Thamsborg, S. M., R. J. Jørgensen, P. J. Waller, and P. Nansen. 1996. The influence of stocking rate on gastrointestinal nematode infections of sheep over a 2-year grazing period. *Veterinary Parasitology* 67:207-224.

Van Houtert, M. F. J, and A. R. Sykes. 1996. Implications of nutrition for the ability of ruminants to withstand gastrointestinal nematode infections. *International Journal of Parasitology* 26:1151-1167.

Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *Journal of Dairy Science* 74:3583-3597.

Van Wyk, J. A., J. Cabaret, and L. M. Michael. 2004. Morphological identification of nematode larvae of small ruminants and cattle simplified. *Veterinary Parasitology* 119:277-306.

Waller, P. J. 2006. Sustainable nematode parasite control strategies for ruminant livestock by grazing management and biological control. *Animal Feed Science and Technology* 126:277-289.

Wang, Z., and L. A. Goonewardene. 2004. The use of MIXED models in the analysis of animal experiments with repeated measures data. *Canadian Journal of Animal Science* 84:1-11

Is the anthelmintic effect of heather supplementation to grazing goats always accompanied by antinutritional effects?

P. Frutos [1] , J. Moreno-Gonzalo [2], G. Hervás [1], U. García [3], L. M. M. Ferreira [4], R. Celaya [3], P. G. Toral [1], L. M. Ortega-Mora [2], I. Ferre [2] and K. Osoro [3]

[1] *Estación Agrícola Experimental, Consejo Superior de Investigaciones Científicas (CSIC), Finca Marzanas 24346 León, Spain*

[2] *SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n 28040 Madrid, Spain*

[3] *Servicio Regional de Investigación y Desarrollo Agroalimentario (SERIDA), Área de Sistemas de Producción Animal, Consejería de Medio Ambiente y Desarrollo Rural, Principado de Asturias, 33300 Villaviciosa, Spain*

[4] *CECAV, Departamento de Zootecnia, Universidade de Trás-os-Montes e Alto Douro, PO Box 1013, 5000-911 Vila Real, Portugal*

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Abstract

To test the hypothesis that the beneficial anthelmintic effect of consuming moderate amounts of tannins may not always be accompanied by anti-nutritional effects in goats, two experiments were conducted. In the first, 48 Cashmere goats were randomly assigned to two treatments: supplementation with tannin-containing heather (6.4% total tannins) or non-supplementation. All goats grazed continuously from May to September under farm conditions in a mountainous area of northern Spain. The mean percentage of heather incorporated into the diet of the supplemented animals was 29.1%. Supplementation reduced the mean number of nematode eggs in faeces ($P < 0.001$) and the goat mortality rate ($P < 0.05$). The rumen ammonia concentration was markedly reduced in the goats receiving the heather supplement (160 vs. 209 mg/l; $P < 0.01$), while volatile fatty acid (VFA) concentrations were significantly greater (63.0 vs. 53.6 mmol total VFA/l; $P < 0.05$). The heather-supplemented goats also showed a lower loss of live weight ($P < 0.01$) and body condition score ($P < 0.001$). In the second experiment, batch cultures of rumen microorganisms with rumen fluid from nine goats whose diet included 29% heather – or not, were used to incubate three substrates (pasture, pasture + heather and pasture + heather + polyethylene glycol) to investigate in vitro ruminal fermentation. Differences ($P < 0.01$) among substrates were observed in terms of dry matter disappearance (DMD), true substrate digestibility (ivTSD), gas production and ammonia concentration, the greatest values always associated with the pasture substrate. Cultures involving rumen inoculum derived from goats receiving the heather-containing diet showed slightly lower DMD (46.9 vs. 48.5 g/100 g; $P < 0.05$), ivTSD (64.6 vs. 65.9 g/100 g; $P < 0.10$) and gas production (105 vs 118 mL/g; $P < 0.001$) values, but much greater total VFA concentrations (48.5 vs. 39.3 mmol/l; $P<0.05$), and suggest the efficiency of ruminal fermentation in these animals was probably improved. Together, the results support the absence of a clear nutritional cost counteracting the beneficial anthelmintic effect of supplementing the diet of grazing goats with tannin-containing heather.

Keywords: gastrointestinal nematode, rumen fermentation, tannin, body weigh

1. Introduction

A growing number of studies relates the consumption of tannin-rich plants by small ruminants with the regulation of their gastrointestinal (GI) nematode populations. This could reduce dependence on conventional chemotherapy, to which many parasite species have developed resistance, and facilitate the sustainable control of gastrointestinal nematode parasitism (Githiori *et al.*, 2006; Hoste *et al.*, 2006). Parasite-infected goats that consume moderate quantities of tannin-containing plants show improved resistance and resilience to parasites, and significant decreases in faecal egg counts (FEC) and parasite burden (Min *et al.*, 2005; Paolini *et al.*, 2005; Osoro *et al.*, 2007a and 2007b). Goats can, however, also experience the anti-nutritional effects of tannins (e.g., Silanikove *et al*., 1997; Ben Salem *et al*., 2003).

It is widely accepted that the consumption of tannins by parasitized animals may result in either favourable or detrimental net effects depending on whether or not the positive anthelmintic action of these plant secondary metabolites outweighs their negative nutritional cost to the host (Houdijk and Athanasiadou, 2003). Nevertheless, some tannins have also been reported as capable of producing a positive nutritional effect when consumed in moderate concentrations, which is associated with the reduction of dietary protein degradation in the rumen (Barry and McNabb, 1999; Mueller-Harvey, 2006).

Earlier studies (Osoro *et al.*, 2007a and 2007b) showed that grazing goats on a diet supplemented with tannin-containing heather had a lower FEC and a better performance, in comparison with animals on non-supplemented diets. On the basis of those studies, we hypothesized that the beneficial anthelmintic effect of the consumption of moderate amounts of tannins might not always be accompanied by marked anti-nutritional effects in goats. Two experiments were conducted to test this hypothesis. The first was performed under farm conditions in a less-favoured mountainous region of Spain where grazing animals are raised extensively and form part of the dynamics of the natural ecosystem. The second experiment involved an *in vitro* assay, with batch cultures of rumen microorganisms, to further investigate the nutritional effects of the inclusion of heather in the diet observed in the first.

2. Materials and methods

Experiments 1 and 2 were performed in accordance with Spanish Royal Decree 1201/2005 for the protection of animals used for experimental and other scientific purposes.

Experiment 1 (*in vivo*)

Experimental site

This experiment was conducted in a mountainous area (altitude 1,000 m) of northwestern Spain (6º 53´ W, 43º 21´ N; Sierra de San Isidro, Illano, Asturias) dominated by shrubby heather-gorse vegetation. Improved pastures were established in 2001 by removing the shrubs present and sowing perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.). A plot of approximately 20,000 m^2 previously managed homogeneously was divided into two identical plots of approx. 10,000 m^2 to accommodate all the animals used in this experiment.

During the experimental grazing season (May-September), the mean monthly rainfall ranged from 48 mm in August to 133 mm in September. The maximum and minimum temperatures were recorded in September (31ºC) and May (2ºC) respectively; the mean average monthly values ranging between 11.8ºC in May and 17.8ºC in July.

Animals and experimental design

Forty-eight lactating Cashmere goats (weighing 36.8 ± 1.13 kg at the beginning of the trial), balanced for live body weight (BW) and body condition score (BCS), were randomly assigned, together with their single kids, to one of two treatments: supplementation with heather (+H) and non-supplementation (−H). Each group was confined to one of the two plots of pasture (containing no heather, as explained above) of approximately 10,000 m^2 for the whole grazing season. The +H goats were offered, in the morning and once every 3 days, freshly cut heather *ad libitum*; the −H goats received no supplement. All animals were reared outdoors under farm conditions (i.e., not preventing nematode infection).

Two weeks before the experiment started, all animals were orally treated against gastrointestinal nematodes with ivermectin (Oramec, Merial, Lyon, France) at about twice the recommended dose (2 mg/kg of BW). The animals were turned out on the pastures on April 19th and left grazing until September 10th.

Sampling procedures and analyses

Pasture. To monitor grass availability, the sward surface height was measured every 10-15 days using an HFRO swardstick (Barthram, 1986), taking 200 measurements at random in each plot. The botanical composition of the pasture was assessed in August using a point quadrant (Grant, 1981) and recording 200 vertical hits per plot. Pasture samples collected in August were analyzed for dry matter (DM; ISO 6496:1999), organic matter (OM; ISO 5984:2002) and crude protein (CP; ISO 5983-2:2005). Neutral and acid detergent fibre (NDF and ADF) and acid detergent lignin (ADL) were determined by the method of Goering and Van Soest (1970) and Van Soest *et al.* (1991).

Heather. The species composition of the heather offered to the goats was assessed in August by recording 1,000 random contacts with the HFRO swardstick over the area where the heather was cut. Green shoots of this heather (less than 3 mm in diameter) were collected for chemical analysis. The nutritive quality (DM, OM, CP, NDF, ADF, and ADL) was analyzed following the same procedures as used to analyze the pasture. The total phenol (TP) and total tannin (TT) contents of the heather offered to the goats were determined using the Folin-Ciocalteu assay in combination with polyvinyl-polypyrrolidone, using tannic acid (Merck, Damstadt, Germany) as the reference standard (Makkar, 2003).

Diet selection. In August, half of the +H animals were randomly chosen and subjected to estimations of the percentage of heather incorporated into the diet using *n*-alkane markers (Ferreira *et al.*, 2005). Samples of pasture, heather, and faeces were collected and calculations performed using a least squares optimization procedure, that minimizes the discrepancies between the actual concentration of alkanes in faeces (adjusted for incomplete faecal recoveries using the recovery values obtained by Ferreira *et al.*, 2005) and the estimated proportion in the diet.

Parasitological procedures. Approximately once per month between May and September, spot samples of goat faeces were individually collected by rectal grab to assess gastrointestinal nematode egg excretion. The number of eggs per gram of faeces (faecal egg count; FEC) were estimated using the modified McMaster technique (MAFF, 1978) with sodium chloride as the flotation medium, in which every egg is regarded as equivalent to 15 eggs per gram (epg) fresh faeces. Faecal cultures were performed for each group by pooling samples from the different individuals as described by the MAFF (1978) and the genus of third-stage nematode larvae identified according to Van Wyk *et al.* (2004).

Body weight and body condition score. All animals were weighed and their BCS assessed on a scale of 1 to 5 (1 = emaciated, 5 = fat; Russel, 1990), at the beginning and end of the experiment and at monthly intervals.

Ruminal fermentation variables. In August, after an overnight fast, a sample of ruminal fluid was individually collected from each goat via a stomach tube and visually checked to ensure that it did not contain saliva. Immediately afterwards each fluid was strained through two layers of gauze and 4 mL were acidified with 4 mL 0.2 N HCl for ammonia determination. A further aliquot of 0.8 mL was added to 0.5 mL of a deproteinizing solution (2% metaphosphoric and 0.4% crotonic acids (wt/vol) in 0.5 N HCl) for determination of volatile fatty acid (VFA). All samples (two per animal and parameter to be analyzed) were stored at −30ºC until analysis. The ammonia concentration was determined by colorimetry and VFA by gas chromatography, using crotonic acid as the internal standard, both in centrifuged samples (Frutos *et al*., 2004).

Mortality rate. Goats were daily checked for clinical signs of illness. When an animal was considered terminally ill, it was humanely euthanized and complete post-mortem examination was performed to discard mortality for other causes.

Statistical analysis

Sward height, FEC, BW and BCS data were analyzed by repeated measures analysis using the MIXED procedure of the Statistical Analysis System program (SAS, 1999). The first measurement in May was used as the covariate. For FEC, BW and BCS, the animals were nested within the treatment to provide the error term to contrast the effect of heather supplementation. Data on FEC were log transformed ($\log_{10}$ x + 1) to normalize their distribution.

The rumen variables were analyzed by one-way analysis of variance, using the GLM procedure. The mortality rate was analyzed using the Chi-squared test (SAS, 1999).

Significant differences were declared at $P < 0.05$ and tendencies at $P < 0.10$.

Table 1. Chemical composition (g/kg of DM, except for DM itself [g/kg of fresh matter]) of feeds and diets used in Experiments 1 and 2.

	DM	OM	CP	NDF	ADF	ADL	TT[1]	TP[1]
Experiment 1 [2]								
Pasture	328	949	143	590	269	25	nd[3]	nd
Heather	423	979	61	557	436	224	64	94
Experiment 2 (diets)[4]								
AGH	865	899	154	591	347	61	nd	nd
AGH+H	776	921	127	581	364	101	23	33

DM = dry matter; OM = organic matter; CP = crude protein; NDF = neutral detergent fibre; ADF = acid detergent fibre; ADL = acid detergent lignin; TT = total tannins; TP = total phenols; [1] g tannic acid equivalents/kg of DM;[2] Samples collected in August; [3] nd = not determined; [4] AGH = alfalfa and grass hays; AGH+H = alfalfa and grass hays (71%) + heather (29%).

Experiment 2 (in vivo - in vitro)

Animals and diets

Nine non-lactating Cashmere goats (28.3 ± 2.30 kg BW) were used in this experiment, which was performed at an experimental research station. Animals were individually fed a mixture of alfalfa and grass hays (AGH; 18 g DM/kg of BW) once a day over a 14-day period. They were then switched to a diet containing 71% of the same mixture of hays and 29% heather (AGH+H; 18 g DM/kg of BW) for 12 more days. The heather was collected from the same area as in Experiment 1. These diets (treatments) were designed on the basis of the outcome of diet selection (see results from Experiment 1), to experimentally simulate the −H and +H treatments.

Table 1 shows the chemical composition of the mixture of grass and alfalfa hays and of the heather used in this experiment.

In vitro *ruminal fermentation*

The effect of the diet on *in vitro* ruminal fermentation was studied in four runs of incubation using a modification of the gas production technique described by Theodorou *et al.* (1994). On day 12 of the experiment, after an overnight fast, ruminal fluid was collected from each animal by stomach tube (as in Experiment 1). The collected samples were transferred to the laboratory in pre-warmed thermos flasks, strained through a double layer of muslin, and kept under CO_2 flushing. The nine inocula were mixed to form a single inoculum (run 1). The whole process was repeated on day 14 to obtain the replicate (run 2). When the goats received the heather-containing diet, rumen fluids were again withdrawn on days 24 (run 3) and 26 (run 4).

Samples of the pasture and heather collected in August during Experiment 1, ground through a 1-mm screen, were used to provide two substrates: pasture (P; 100% pasture) and pasture + heather (P+H; 71% pasture and 29% heather). A third substrate (P+H+PEG) was prepared by adding one gram per flask of a tannin-binding agent (polyethylene glycol, PEG, MW 6000; Fluka Chemie GmbH, Buchs, Switzerland) - which inhibits the effect of tannins (Makkar, 2003)- to the P+H substrate. The large quantity of PEG was chosen because the efficiency of this agent is believed to depend on the tannins/PEG ratio, and to avoid the possibility that too small an amount of this inert compound might be associated with a lack of effect.

Each incubation run included a total of twelve 125 mL sealed serum flasks. Three samples of each substrate (≈ 500 mg) and three blanks (i.e., buffered rumen fluid without substrate) were incubated at 39ºC with 10 mL rumen fluid and 40 mL phosphate-bicarbonate buffer. The buffer solution was prepared as described by Goering and Van Soest (1970), with the exception that no trypticase was added.

Gas production was determined by measuring head space gas pressure at 24 h post-inoculation. Pressure values, corrected for the quantity of substrate OM incubated and gas released from the blanks, were used to generate gas volume estimates using a predictive equation derived from earlier simultaneous pressure and volume measurements (Hervás *et al.*, 2005). Immediately afterwards, the fermentations were ended by swirling the bottles on ice. The samples were then centrifuged at 976 × *g* for 10 min and aliquots of the supernatant collected for ammonia and VFA determinations as in Experiment 1.

In vitro DM disappearance (DMD; g/kg) and true substrate digestibility (ivTSD) after 24 h of incubation were estimated by filtering the residues using pre-weighed sintered glass crucibles (100–160 µm; Pyrex, Stone, UK) and determining the NDF content, as reported in Frutos *et al.* (2004).

Chemical analyses

All chemical analyses were performed as in Experiment 1.

Statistical analysis

Data for *in vitro* ruminal variables were analyzed by one-way analysis of variance, using the GLM procedure of the Statistical Analysis System program (SAS, 1999) to examine the effects of the two main sources of variation, goat diets and substrates, as well as their interaction. The mean value of each set of three flasks per substrate and run was considered as the experimental unit. Significance was set at $P < 0.05$; trends were declared at $P < 0.10$.

3. Results

Experiment 1

Sward height, and pasture and heather botanical and chemical composition

As shown in Figure 1, the available sward height differed significantly between treatments, with greater values being recorded in the pasture used by the +H animals ($P < 0.001$). Mean sward height gradually fell from 10.2 cm at the beginning of the experiment (May) to 3.7 cm at the end (September) ($P < 0.001$).

The botanical composition of the available pasture in August was on average 6.8% white clover, 21.9% perennial ryegrass, 43.1% other grasses (mainly *Agrostis capillaris* L.), and 27.9% dead matter, with no differences between treatments. The chemical composition of the pasture samples taken in August showed a CP content of 143 g/kg of DM, and values for NDF, ADF and ADL of 590, 269 and 25 g/kg of DM, respectively (Table 1).

The heather offered was mostly composed of *Calluna vulgaris* (L.) Hull (61%), with 25% *Erica umbellata* L., 12% *E. cinerea* L., 1% *E. tetralix* L., and 1% *Daboecia cantabrica* (Hudson) C. Koch. Chemical composition analysis showed a lower CP content (61 g/kg of DM) and greater cell wall content (557, 436 and 224 g/kg of DM for NDF, ADF and ADL, respectively), as expected for shrub species. Total phenol and total tannin contents were estimated to be 94 and 64 g tannic acid equivalents/kg of DM, respectively (Table 1).

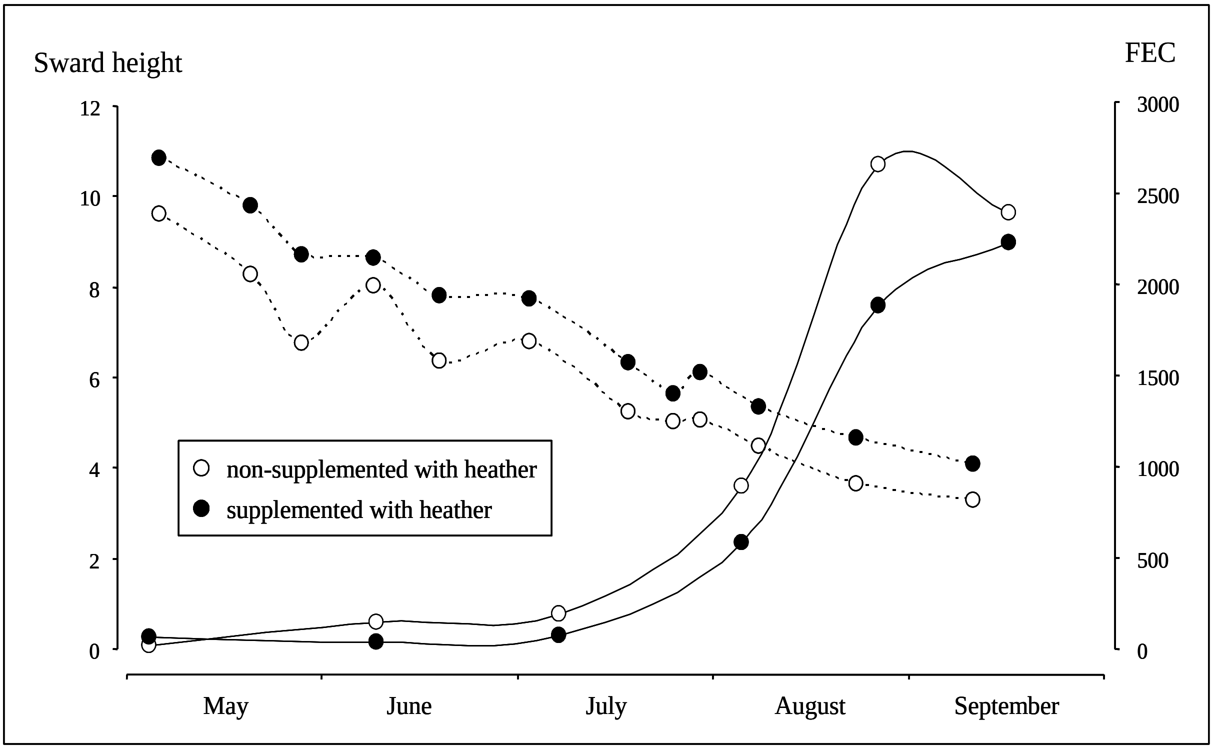

Figure 1. Faecal gastrointestinal nematode egg counts (FEC; eggs/g; solid lines —) and sward height (cm; short-dash lines ---) over the experimental season in grazing goats whose diets were supplemented - or not - with heather. Mean log transformed FEC values = 2.20 vs. 2.66 eggs/g for supplemented *vs.* non-supplemented animals (sed = 0.115; $P < 0.001$), and mean sward heights = 6.8 vs. 5.7 cm for supplemented vs non-supplemented animals (sed = 0.07; $P < 0.001$).(sed = standard error of the differences of means)

Diet composition

The mean percentage of heather incorporated into the diet of the +H animals was 29.1% (± 2.06) in August. The remaining 70.9% corresponded to herbaceous species.

Faecal egg counts and goat mortality

Supplementation with heather reduced the mean FEC ($P < 0.001$; see Figure 1); values ranged from 15 epg at the beginning of the trial to a maximum of 2653 at the end of August. The −H goats with the greatest FEC in the two samplings performed in August died before the September sampling (seven in total), which explains the reduction seen in Figure 1. Supplementation with heather significantly reduced the mortality rate ($P < 0.05$): at the end of the grazing period, two +H and eight –H does had to be euthanized.

The nematode genera identified were *Trichostrongylus*, *Teladorsagia*, and *Oesophagostomum*. *Trichostrongylus* was the predominant genus (60−95%) over the entire experimental period; *Teladorsagia* and *Oesophagostomum* were detected in lower percentages (5−25%).

Table 2. Body weight (BW, kg) and body condition score (BCS) during the experimental grazing period (from May to September) in goats supplemented (+H) or not (−H) with heather

	BW			BCS		
	−H	+H	SEM[1]	−H	+H	SEM
May	37.0	36.3	1.12	2.57	2.56	0.042
June	34.5	34.2	0.89	2.57	2.59	0.043
July	36.2[b]	37.7[a]	0.92	2.53[b]	2.85[a]	0.049
August	30.3[b]	32.4[a]	0.83	2.17[b]	2.38[a]	0.068
September	29.7[b]	30.7[a]	0.87	1.45[b]	2.00[a]	0.076
P - value[2]						
Treatment		< 0.01			< 0.01	
Date of sampling		< 0.01			< 0.01	
Treatment x date		< 0.01			< 0.01	

[1] SEM = standard error of the mean; [2] Probability value; [a,b] For each variable, means in a row with a different superscript differ significantly ($P<0.05$).

Live weight and body condition changes

Table 2 shows that heather supplementation was responsible for the smaller losses of live body weight ($P < 0.01$) and body condition score ($P < 0.01$) recorded over the experimental grazing season in the +H animals.

Ruminal fermentation variables

Table 3 shows the ammonia and VFA concentrations in the ruminal fluid of the experimental animals. The rumen ammonia concentration was markedly reduced in the goats receiving tannin-containing heather ($P < 0.01$). On the contrary, total VFA concentration was significantly greater in these animals ($P < 0.05$), mainly due to an increase in acetic acid (approx. 26%) but also in propionic acid (approx. 16%). The concentrations of valerate and branched-VFAs (referred to as “others” in Table 3), however, decreased. The molar proportions of VFA (data not shown) were also modified by supplementation with heather ($P < 0.001$), with increases in acetate and reductions in butyrate, valerate, and branched-VFA.

Experiment 2

As shown in Table 4, significant differences in gas production, dry matter disappearance, *in vitro* true substrate digestibility and ammonia and volatile fatty acid concentrations were observed due to both goat diet (i.e., including or not heather: AGH vs. AGH+H) and substrates incubated. However, no significant interaction (diet x substrate) was found for any *in vitro* rumen fermentation variables ($P > 0.10$).

Cultures with rumen inoculum derived from goats receiving the heather containing diet (AGH+H) showed slightly lower values of DMD (-3%), ivTSD (-2%) and gas production (-11%) compared to cultures from AGH animals. On the contrary, those incubations (AGH+H) returned greater values of total VFA (23%), mainly accounted for by greater concentrations of acetate (34%) and butyrate (21%). The molar proportions of VFA (data not shown) tended to be modified by the incorporation of heather into the diet ($P < 0.10$), with increases in acetate and reductions in propionate, and valerate and branched-VFAs (referred to as “others” in Table 4). Differences among substrates ($P < 0.01$) were observed in terms of gas production, DMD, ivTSD and ammonia concentrations, the greatest values always being found for the pasture (P) substrate and the lowest for the P+H substrate. When PEG was added to the mixture (P+H+PEG), gas production and ivTSD were

Table 3. Ammonia (mg/l) and volatile fatty acid (VFA, mmol/l) concentrations in the ruminal fluid of grazing goats whose diets were supplemented (+H) or not supplemented (−H) with heather

	−H	+H	SEM[1]	*P* – value[2]
Ammonia	209.4[a]	159.7[b]	9.95	0.01
Total VFA	53.61[b]	62.98[a]	2.340	0.02
Acetate	36.11[b]	45.47[a]	1.845	0.01
Propionate	8.80[b]	10.25[a]	0.500	0.07
Butyrate	5.48	5.08	0.270	0.34
Others[3]	3.22[a]	2.17[b]	0.137	0.01

[1] SEM = standard error of the mean;[2] Probability value;[3] Calculated as the sum of valerate, isovalerate, isobutyrate and caproate; [a,b] Means in a row with a different superscript differ significantly ($P<0.05$).

significantly increased. However, the addition of PEG did not significantly inhibit the negative effect of heather on DMD and ammonia concentration.

No significant differences among substrates were observed for the concentration of any particular VFA.

4. Discussion

The control of GI parasites with anthelmintics is becoming increasingly difficult worldwide due to drug resistance and concerns over pesticide residues in the food chain and the general environment (Van Houtert and Sykes, 1996). Thus, the greater variety of control methods used in combination, the longer we can expect to have effective worm control. The use of tannin-containing plants has proven effective in the control of GI parasite infections, as confirmed in this study where supplementing the diets of grazing goats with tannin-containing heather significantly reduced faecal egg counts (see Figure 1). This is in line with previous reports for the same area by Osoro *et al.* (2007a and 2007b). However, the mean FEC values (as high as 2652 epg) were notably different from those found in the above papers, which is almost certainly related to the effect of climatic conditions on parasite infections (Stromberg, 1997).

Several authors have reported that the intake of low to moderate quantities of condensed tannins (CTs) may have beneficial effects on parasitized ruminants. For example, we reported reductions in FEC of up to 75%, compared to control goats, in an experiment that included supplementation with a heather containing between 7 and 8.6% tannin (expressed in tannic acid equivalents; Osoro *et al.*, 2007b). Butter *et al.* (2000) observed a reduction in FEC when 5% of a commercial extract of quebracho (*Schinopsis* spp.) CTs were included in a low protein diet. Min *et al.* (2005) reported a great reduction in FEC when does were fed *Sericea lespedeza* (*Lespedeza cuneata*) containing 15.2% tannins (using an internal standard from the plant itself as the reference).

In the present study, FEC reduction was achieved with the dietary incorporation of an estimated 29% heather containing 6.4% tannin (which may be considered a low-to-moderate intake of tannins). The lowest dietary tannin threshold needed for anthelmintic effects remains unclear, however. Moreover, it should be remembered that different plants contain tannins with different structures and hence reactivities, so the notion of a "low-to-moderate" content could be misleading. In addition, different research groups use different methods to analyse this large group of phenolic compounds, but the equivalents in which the tannin content are expressed (*e.g.*, tannic acid, internal standards extracted from the plants themselves, etc.) are not always given, which means that comparisons cannot be made (Makkar, 2003; Álvarez del Pino *et al.*, 2005).

A number of investigations have focused on the potential use of CTs to control GI parasitism because these are relatively stable in the digestive tract of ruminants and rarely have toxic effects (Mueller-Harvey,

Table 4. Gas production (mL/g OM), dry matter disappearance (DMD; g/100 g), in vitro true substrate digestibility (ivTSD; g/100 g), ammonia (mg/l) and VFA concentration (mmol/l) in batch cultures after 24 h incubation

	Goat diet[1]			Substrate[2]				*P* – value[4]		
	AGH	AGH+H	SEM[3]	P	P+H	P+H+PEG	SEM	Diet	Substrate	diet x subst
Gas production	118.8[a]	105.2[b]	1.55	123.5[a]	102.1[c]	110.4[b]	1.903	< 0.01	< 0.01	0.39
DMD	48.48[a]	46.94[b]	0.358	52.82[a]	44.40[b]	45.90[b]	0.439	0.03	< 0.01	0.55
ivTSD	65.95[a]	64.61[b]	0.431	67.23[a]	63.08[b]	65.54[a]	0.775	0.07	< 0.01	0.82
Ammonia	287.7	284.7	3.51	308.6[a]	269.4[b]	280.74[b]	4.30	0.57	< 0.01	0.31
Total VFA	39.27[b]	48.45[a]	1.830	45.44	41.79	44.34	2.241	0.02	0.54	0.97
Acetate	23.65[b]	31.81[a]	1.333	28.91	26.83	27.43	1.632	< 0.01	0.67	0.82
Propionate	10.44	10.82	0.746	10.70	9.99	11.22	0.913	0.74	0.66	0.81
Butyrate	3.01[b]	3.65[a]	0.208	3.56	3.10	3.33	0.255	0.08	0.48	0.97
Others[5]	2.17	2.18	0.126	2.28	1.88	2.36	0.155	0.97	0.15	0.88

[1] AGH = alfalfa and grass hays; AGH+H = alfalfa and grass hays (71%) + heather (29%);[2] P = pasture; P+H = pasture (71%) + heather (29%); PEG = polyethylene glycol; [3] SEM = standard error of the mean; [4] Probability value; [5] Calculated as the sum of valerate, isovalerate, isobutyrate and caproate;[a,b] For each source of variation (goat diet or substrate), means in a row with a different superscript differ significantly (*P*<0.05)

2006). CTs may affect protein nutrition by reducing dietary protein degradation in the rumen, thus enhancing the amount of protein available for digestion in the small intestine (Barry and McNabb, 1999; Frutos *et al.*, 2000; Mueller-Harvey, 2006). According to Coop and Kyriazakis (2001), protein nutrition, and therefore CTs, may influence the development and consequences of parasitism by increasing the resilience and resistance of the host or by directly affecting the parasite populations.

Given the low content of protein in the offered heather (61 g/kg of DM), it seems very unlikely that the anthelmintic effect of its supplementation would be due to a greater availability of protein, although there might be a greater availability of non-degradable protein due to the effect of the tannin (Barry and McNabb, 1999). A common characteristic of GI parasitism is an increased loss of endogenous protein to the GI tract (Coop and Kyriazakis, 2001) and some authors have suggested that the inclusion of CTs might be an alternative to providing high protein rations as a means of reducing the consequences of parasitism (Van Houtert and Sykes, 1996; Butter *et al.*, 2000; Coop and Kyriazakis, 2001).

The supplementation with tannin-containing heather significantly reduced the goat mortality rate, which is consistent with previous studies reporting the positive effect of tannin consumption on the resilience of goats (Paolini *et al.*, 2005).

In addition to the effect of heather supplementation and due to the differences observed between treatments in the sward height, it cannot be completely ruled out that the –H goats consumed more contaminated pasture than +H goats. A shorter sward may have larger parasite burdens because it is easier for the parasites to migrate to the top (Niezen *et al.*, 1998), so the non-supplemented animals might be at a higher risk of ingestion of nematode infective larvae. Nevertheless, as both experimental plots had the same previous management, they presumably had a similar parasitic status at the start of the trial.

As observed in earlier work (Osoro *et al.*, 2007a and 2007b), the negative BW gains agree with the relationship established between green sward height and BW change (Merchant and Riach, 1994). Considerable BW losses were observed after July, when the sward height fell below 6−7 cm (see Figure 1), probably due to the limited availability of pasture and the increase in parasite burden during the grazing season (Coop and Kyriazakis, 2001). However, losses were mitigated by the effect of heather intake. Houdijk and Athanasiadou (2003) reported evidence suggesting that the consumption of CT-rich plants can result in reduced parasitism and improved performance in parasitized small ruminants, which would probably be the outcome of indirect nutritional benefits (*e.g.*, extra protein availability for the parasitized grazing hosts). On the other hand, those authors also suggest that the net benefit in the performance of parasitized hosts might be the result of the anti-parasitic effects of CTs outweighing their anti-nutritional effects.

The performance results found in this study were supported by the nutritional outcomes. Although the results on ruminal fermentation obtained *in vitro* and *in vivo* were not exactly the same, as expected, they followed a very similar pattern and support the general conclusion that the dose of tannins ingested with the heather diet was insufficient to adversely affect the majority of the rumen microbial populations in goats adapted to its consumption.

Several authors have indicated that dietary concentrations of <50 g CT/kg DM are nutritionally beneficial (these recommendations originated mainly from feeding trials with *Lotus* species and cannot be directly applied to other feeds; Mueller-Harvey, 2006). However, erroneous generalizations have persisted since the first reviews in the 1960s and 1970s stated that tannins are harmful or toxic to mammals (Mueller-Harvey, 2006). The main benefit of tannins in ruminant nutrition stems from their negative effect on proteolysis and the first consequence is a lower concentration of ammonia in the ruminal fluid, as observed in our *in vivo* and *in vitro* approaches. The differences between assays 1 and 2 may be due to the fact that, in *in vitro* studies, lower rumen fluid ammonia concentrations can only be attributed to lower concentrations of protein in the substrate, a reduced deamination of amino acids or a greater rate of uptake by bacteria. In addition, differences may also be explained because in the *in vitro* experiment 29% of the pasture was replaced by exactly the same amount of heather (while substitution in the *in vivo* grazing trial was probably incomplete), and because the response of the ruminal microorganisms to tannins may not be exactly the same *in vivo* and *in vitro*. The fact that no significant differences in ammonia concentration were observed between substrates P+H and P+H+PEG suggests other factors, further to tannins, may also be at work (*e.g.*, the lower nutritive value of P+H compared to P). In the grazing experiment, a significant and negative correlation was found between the percentage of heather incorporated into the diet and the rumen ammonia concentration ($r = -0.60$; $P < 0.05$).

As well known, VFAs are the most important by-products of fermentation and the major energy source of ruminants. The higher concentrations of VFAs for the supplemented goats in both trials (17% and 23%, respectively) are in line with the better performance (BW and BCS) of these animals. The fact that no significant differences were found in total VFA concentration between substrates, but were found when comparing goat diets, suggests an adaptation of rumen microorganisms to the consumption of these phenolic compounds (Smith *et al.*, 2005) and a subsequently better digestive utilization of the diet.

The direct effects reported for tannins on rumen VFAs are not consistent and different authors have indicated different results (*e.g.*, Makkar *et al.*, 1995; Hervás *et al.*, 2003); these discrepancies probably depend on the amount and type of tannins and on the animal species that ingest them. Results from experiments 1 and 2 revealed a significant increase in acetate production when heather was present, as well as differences in the fermentation pathways, reflected in the different molar proportions. The increase in acetate and the reduction in gas production may be accounted for by an effect of tannins on acetateutilizing rumen bacteria, which is probably related to the beneficial and known inhibition of methane-producing microorganisms by these secondary compounds (Tavendale *et al.*, 2005). These results support the improved efficiency of ruminal fermentation in animals consuming tannin-containing heather and confirm those reported in a previous grazing experiment performed under similar conditions (Osoro *et al.*, 2007b).

Comparisons of the results for goats adapted *versus* non-adapted to the consumption of heather (AGH vs. AGH+H; Table 4) show the latter to be associated with reductions in gas production, DMD and ivTSD (in accordance with that widely reported for diets containing tannins; Frutos *et al.*, 2004; Mueller- Harvey, 2006) of a lower magnitude than the improvements in the mean indicators for the utilization of nitrogen or energy (ammonia and VFA concentrations).

In conclusion, the present results suggest there is no substantial nutritional cost counteracting the anthelmintic effect of supplementing the diets of grazing goats with heather, and they therefore support the hypothesis under test. Further research, including the estimation of total pasture and heather intake, would help explain the effects of heather supplementation on nutrition and performance in grazing goats. Eventually, it would also contribute to the development of a sustainable method for controlling GI nematode parasitism in extensive goat production systems.

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References

Álvarez del Pino MC, Hervás G, Mantecón AR, Giráldez FJ and Frutos P 2005. Comparison of biological and chemical methods, and internal and external standards, for assaying tannins in Spanish shrub species. Journal of the Science of Food and Agriculture 85, 583-590.

Barry TN and McNabb WC 1999. The implications of condensed tannins on the nutritive value of temperate forages fed to ruminants. British Journal of Nutrition 81, 263-272.

Barthram GT 1986. Experimental techniques: the HFRO swardstick. The Hill Farming Research Organisation Biennial Report 1984-85, pp. 29-30. HFRO, Bush Estate, Penicuik, Midlothian, UK.

Ben Salem H, Ben Salem I, Nefzaoui A and Ben Saïd MS 2003. Effect of PEG and olive cake feed blocks supply on feed intake, digestion, and health of goats given kermes oak (*Quercus coccifera* L.) foliage. Animal Feed Science and Technology 110, 45-59.

Butter NL, Dawson JM, Wakelin D and Buttery PJ 2000. Effect of dietary tannin and protein concentration on nematode infection (*Trichostrongylus colubriformis*) in lambs. Journal of Agricultural Science 134, 89-99.

Coop RL and Kyriazakis I 2001. Influence of host nutrition on the development and consequences of nematode parasitism in ruminants. Trends in Parasitology 17, 325-330.

Ferreira LMM, Oliván M, García U, Rodrigues MAM and Osoro K 2005. Validation of the alkane technique to estimate diet selection of goats grazing heather-gorse vegetation communities. Journal of the Science of Food and Agriculture 85, 1636-1646.

Frutos P, Hervás G, Giráldez FJ, Fernández M and Mantecón AR 2000. Digestive utilization of quebracho-treated soya bean meal in sheep. Journal of Agricultural Science 134, 101-108.

Frutos P, Hervás G, Giráldez FJ and Mantecón AR 2004. An *in vitro* study on the ability of polyethylene glycol to inhibit the effect of quebracho tannins and tannic acid on rumen fermentation in sheep, goats, cows and deer. Australian Journal of Agricultural Research 55, 1125-1132.

Githiori JB, Athanasiadou S and Thamsborg SM 2006. Use of plants in novel approaches for control of gastrointestinal helminths in livestock with emphasis on small ruminants. Veterinary Parasitology 139, 308-320.

Goering MK and Van Soest PJ 1970. Forage fiber analysis (apparatus, reagents, procedures and some applications). Agric. Handbook No. 379, ARS-USDA, Washington, DC, USA.

Grant SA 1981. Sward components. In Sward measurement handbook (eds. J Hodgson, RD Baker, A Davies, AS Laidlaw and JD Leaver), pp. 71–92. British Grassland Society, Hurley, Maidenhead, Berkshire, UK.

Hervás G, Frutos P, Giráldez FJ, Mantecón AR and Álvarez del Pino MC 2003. Effect of different doses of quebracho tannins extract on rumen fermentation in ewes. Animal Feed Science and Technology 109, 65-78.

Hervás G, Frutos P, Giráldez FJ, Mora MJ, Fernández B and Mantecón AR 2005. Effect of preservation on fermentative activity of rumen fluid inoculum for *in vitro* gas production techniques. Animal Feed Science and Technology 123-124, 107-118.

Hoste H, Jackson F, Athanasiadou S, Thamsborg SM and Hoskin SO 2006. The effects of tannin-rich plants on parasitic nematodes in ruminants. Trends in Parasitology 22, 253-261.

Houdijk JGM and Athanasiadou S 2003. Direct and indirect effects of host nutrition on ruminant gastrointestinal nematodes. In Matching herbivore nutrition to ecosystems biodiversity (eds. L 't Mannetje, L Ramírez, C Sandoval-Castro and JC Ku-Vera), pp. 213–236. VI Int. Symp. Nutrition of Herbivores, Univ. Aut. Yucatán, Mérida, México.

MAFF 1978. Manual of veterinary parasitological techniques. Tech. Bull. No. 18. Ministry of Agriculture, Fisheries and Food, London, UK.

Makkar HPS 2003. Quantification of tannins in tree and shrub foliage. A laboratory manual. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Makkar HPS, Becker K, Abel H and Szegletti C 1995. Degradation of condensed tannins by rumen microbes exposed to quebracho tannins (QT) in rumen simulation technique (RUSITEC) and effects of QT on fermentative processes in the RUSITEC. Journal of the Science of Food and Agriculture 69, 495-500.

Merchant M and Riach DJ 1994. The intake and performance of Cashmere goats grazing sown swards. Grass and Forage Science 49, 429-437.

Min BR, Hart SP, Miller D, Tomita GM, Loetz E and Sahlu T 2005. The effect of grazing forage containing condensed tannins on gastro-intestinal parasite infection and milk composition in Angora does. Veterinary Parasitology 130, 105-113.

Mueller-Harvey I 2006. Unravelling the conundrum of tannins in animal nutrition and health. Journal of the Science of Food and Agriculture 86, 2010-2037.

Niezen JH, Charleston WAG, Hodgson J, Miller CM, Waghorn TS, Robertson HA 1998. Effect of plant species on the larvae of gastrointestinal nematodes which parasitise sheep. International Journal for Parasitology 28, 791-803.

Osoro K, Benito-Peña A, Frutos P, García U, Ortega-Mora LM, Celaya R and Ferre I 2007a. The effect of heather supplementation on gastrointestinal nematode infections and performance in Cashmere and local Celtiberic goats on pasture. Small Ruminant Research 67, 184-191.

Osoro K, Mateos-Sanz A, Frutos P, García U, Ortega-Mora LM, Ferreira LMM, Celaya R and Ferre I 2007b. Anthelmintic and nutritional effects of heather supplementation on Cashmere goats grazing perennial ryegrass-white clover pastures. Journal of Animal Science 85, 861-870.

Paolini V, De La Farge F, Prevot F, Dorchies Ph and Hoste H 2005. Effects of the repeated distribution of sainfoin hay on the resistance and the resilience of goats naturally infected with gastrointestinal nematodes. Veterinary Parasitology 127, 277-283.

Russel AJF 1990. Body condition scoring of goats. Scottish Cashmere Producers Association Newsletter 10, 3.

SAS 1999. SAS OnlineDoc®, Version 8, SAS Institute Inc., Cary, NC, USA.

Silanikove N, Gilboa N and Nitsan Z 1997. Interactions among tannins, supplementation and polyethylene glycol in goats given oak leaves: effects on digestion and food intake. Animal Science 64, 479-483.

Smith HA, Zoetendal E and Mackie RI 2005. Bacterial mechanisms to overcome inhibitory effects of dietary tannins. Microbial Ecology 50, 197-205.

Stromberg BE 1997. Environmental factors influencing transmission. Veterinary Parasitology 72, 247-264.

Tavendale MH, Meagher LP, Pacheco D, Walker N, Attwood GT and Sivakumaran S 2005. Methane production from in vitro rumen incubations with *Lotus pedunculatus* and *Medicago sativa*, and effects of extractable condensed tannin fractions on methanogenesis. Animal Feed Science and Technology 123-124, 403-419.

Theodorou MK, Williams BA, Dhanoa MS, McAllan AB and France J 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Animal Feed Science and Technology 48, 185-197.

Van Houtert MFJ and Sykes AR 1996. Implications of nutrition for the ability of ruminants to withstand gastrointestinal nematode infections. International Journal of Parasitology 26, 1151-1167.

Van Soest PJ, Robertson JB and Lewis BA 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysacharides in relation to animal nutrition. Journal of Dairy Science 74, 3583-3597.

Van Wyk JA, Cabaret J and Michael LM 2004. Morphological identification of nematode larvae of small ruminants and cattle simplified. Veterinary Parasitology 119, 277-301

CAPÍTULO III

Infecciones experimentales

Anthelmintic effect of heather supplementation in Cashmere goats experimentally infected with *Trichostrongylus colubriformis*

Javier Moreno-Gonzalo[1], Koldo Osoro[2], Urce García[2], Pilar Frutos[3], Rafael Celaya[2], Luis MM Ferreira[4], Luis Miguel Ortega-Mora[1] and Ignacio Ferre[1*]

Author details

[1] SALUVET Group, Department of Animal Health, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040-Madrid, Spain. [2] Servicio Regional de Investigación y Desarrollo Agroalimentario (SERIDA), Área de Sistemas de Producción Animal, 33300-Villaviciosa, Asturias, Spain. [3] Instituto de Ganadería de Montaña, CSIC-ULE, Finca Marzanas, 24346-León, Spain. [4] CECAV, Departamento de Zootecnia, Universidade de Trás-os-Montes e Alto Douro, PO Box 1013, 5000-911 Vila Real, Portugal

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Abstract

Background: Recently, the anthelmintic effect of heather consumption has been studied in grazing goats. In the present experiment the effects of heather consumption on the establishment of incoming infective larvae (experiment 1, preventive treatment) and an adult worm population (experiment 2, curative treatment) were investigated in Cashmere goats experimentally infected with *Trichostrongylus colubriformis*. In experiment 1, 12 castrated male goats were divided into 2 groups: heather-supplemented *vs.* non-supplemented animals. After 2 weeks of adaptation to the diet, all goats were experimentally infected *per os* with 6,000 *T. colubriformis* third stage larvae (L3). Three weeks post-infection (pi), the goats were slaughtered, and worm counts as well as female worm fecundity and development were determined. In experiment 2, 15 non-lactating does were experimentally infected with 6,000 *T. colubriformis* L3. At 6 weeks pi, 3 groups were established: control, heather-supplemented and heather-supplemented with polyethylene glycol (PEG). Individual faecal nematode egg output was measured twice weekly to assess gastrointestinal nematode egg excretion. The goats were slaughtered 5 weeks after heather administration (11 weeks pi), and worm counts as well as female worm fecundity and development were subsequently determined.

Results: In experiment 1, heather consumption was associated with a close to significant (P=0.092) reduction in larvae establishment. No effect on fecundity was observed, but the length of female worms in supplemented goats was greater (P<0.001). In experiment 2, heather administration was also associated with a decrease (P<0.001) in egg excretion from 45 to 76 days pi. Although worm counts and female fecundity were lower in supplemented goats, no significant differences were observed.

Conclusions: Overall, the results showed a reduction in *T. colubriformis* larvae establishment and a decrease in nematode egg excretion when heather was administered in experimentally infected goats. The heather plus PEG treatment reduced nematode egg excretion levels at the same proportion as heather, thereby suggesting that the threshold of tannins required for an anthelmintic effect is most likely quite low.

Keywords: *Trichostrongylus colubriformis*, Goat, Heather, Tannin, Polyethylene glycol, Control.

1. Background

Gastrointestinal nematode infections remain one of the principal constraints limiting the productivity and sustainability of pasture-based small ruminant production systems. The use of bioactive plants with anthelmintic properties, especially tannin-rich plants, has been proposed as an alternative or complement for the control of gastrointestinal nematodes in sheep and goats to avoid or delay the development of anthelmintic-resistant nematode strains and to reduce the dependence on chemical anthelmintic treatments [1,2]. Condensed tannins have been reported to benefit ruminant nutrition by both increasing post-ruminal protein availability and reducing the consequences of digestive nematode parasitism [3]. Recently, a number of reports have been published on the anthelmintic effects of tannin-rich plants and forages in small ruminants [4-10]. These studies have shown a decrease in the establishment of third-stage nematode larvae (L3) or a reduction in worm fertility and egg output in naturally infected goats consuming a moderate concentration of tannin-rich plants [1,11-14].

The studies on the utilization of heather supplementation in grazing goats have shown a reduction in the levels of gastrointestinal nematode egg excretion associated with a decrease in worm fertility and/or a reduction in the establishment of incoming L3 as well as an apparent greater resilience of goats against gastrointestinal nematode infections [4,9,15-17]. However, some limitations associated with those trials should be addressed, such as the lack of heather consumption recorded per animal, the ignorance of total worm burden and nematode stages involved, and/or the strong dependence of weather conditions in the gastrointestinal nematode infection dynamics. Additionally, as these studies were conducted in grazing conditions it was not possible to distinguish whether the reduced worm burden in heather-supplemented goats was due to the reduced degree of pasture contamination (because of the lower levels of egg excretion in faeces) or the direct effect of heather consumption on the mortality and fertility of parasites. Recently, anthelmintic effects of 3 Ericaceae (*Calluna vulgaris*, *Erica cinerea* and *Erica umbellata*) on different *T. colubriformis* development stages has been shown *in vitro* (Moreno-Gonzalo et al., unpublished observations).

It has been suggested that the effects associated with the consumption of tannin-rich plants, such as heather, may vary depending on the nematode parasitic stages exposed to the tannins [18]. Thus, 2 indoor experimental trials were performed to assess the effects of heather administration on incoming *T. colubriformis* L3 (preventive treatment) and the influence of heather administration on an established population of *T. colubriformis* (curative treatment). The potential activity of the tannins on a *T. colubriformis* patent infection in goats feeding on heather with or without polyethylene glycol (PEG), an adsorbent that deactivates dietary tannins, was also investigated to help to understand the function of these phenolic compounds in the anthelmintic activity observed. The main objective of the present study was to investigate the anthelmintic effects of heather consumption in Cashmere goats experimentally infected with *Trichostrongylus colubriformis* under controlled conditions.

2. Methods

Experimental site

The experiments were conducted at "El Carbayal" research station in the northwest of Spain (6° 53´ W, 43° 20´ N, Sierra de San Isidro, Illano, Asturias) and carried out in accordance with Spanish Royal Decree 1201/2005 (based on European Council Directive 86/609/ECC) for the protection of animals used for experimental and other scientific purposes.

Heather

Heather was cut every 3 days in a nearby heathland area belonging to the experimental farm and stored chilled until it was administered to the animals. Manual separation of heather samples revealed that it was composed primarily of *Calluna vulgaris* (>90%) with a smaller content of *Erica umbellata* and *Erica cinerea*.

Infective larvae

T. colubriformis (INRA, goat strain, France) L3 were obtained from a donor goat infected with a pure strain of nematode (single infection *per os* with 6,000 L3). Batches of 1-month-old larvae were used in the assays.

Experimental designs

Experiment 1: Preventive administration of heather. Twelve, 45-60-months-old, castrated male Cashmere goats, which were de-wormed with ivermectin (Oramec, Merial, Lyon, France) 4 weeks before the experiment start date, were used for the experiments. The goats showed <100 trichostrongyle eggs per gram (epg) of feces prior to anthelmintic treatment, and no fecal egg counts were recorded when the experiment began. The trial lasted for 5 weeks and was divided into 2 successive periods: a 2-week period for adaptation to the diet and a 3-week experimental period. The goats were divided into 2 balanced groups of 6 goats each according to their body weight (53.4 ± 2.46 kg): heather-supplemented (70% alfalfa hay + 30% heather) *vs*. non-supplemented (alfalfa hay). The diets (30 g DM kg $BW^{-0.75}$ day^{-1}) were offered twice a day (at 9:00 h and 18:00 h). After 2 weeks of adaptation to the diets, all goats were experimentally infected *per os* with a single dose of 6,000 *T. colubriformis* L3. During the experimental period, the goats were maintained in individual metabolic cages with unrestricted water, and their feed intake was monitored. All animals were slaughtered at 3 weeks post-infection (pi), the worms were counted, and female worm fecundity and development were determined.

Experiment 2: Curative administration of heather. Fifteen, 30-50-months-old, non-lactating Cashmere does, weighing 44.1 (± 1.49) kg and de-wormed 4 weeks before experiment start date with ivermectin (Oramec, Merial, Lyon, France), were used. Before anthelmintic treatment, the goats displayed <100 trichostrongyle eggs per gram (epg) of feces, and no fecal egg counts were registered when the experiment began. The trial lasted for 11 weeks and was divided into 2 successive periods: a 6-week period for the establishment of an adult nematode population and a 5-week experimental period including the administration of heather in the supplemented group. All does were experimentally infected *per os* with a single dose of 6,000 *T. colubriformis* L3, and maintained on a concrete floor pen and fed with alfalfa hay *ad libitum*. Six weeks pi, 3 groups of goats were established according to BW and the levels of nematode egg excretion and assigned to 3 different treatments: heather-supplemented, heather-supplemented plus PEG, and control non-supplemented. The animals were located in individual metabolic cages with unrestricted water to monitor feed and faeces output. The diets were established as in experiment 1. Goats of the heather plus PEG group orally received 35 g of PEG 6000 (Fluka Chemie GmbH, Buchs, Switzerland) dissolved in 70 mL of water (animals of the other groups received an equal volume of water daily) every day immediately before the morning meal. Individual faecal egg count (FEC) was measured twice weekly. The goats were slaughtered after 5 weeks of heather administration (11 weeks pi) for parasitological studies including total worms counting as well as female worm fecundity and development determination.

Tannin content in heather

Assays for heather total phenolics and tannins were conducted following the Folin-Ciocalteu technique in combination with polyvinylpolypyrrolidone using tannic acid (Merck, Damstadt, Germany) as the reference standard [19]. The values are expressed in g tannic acid equivalents per kg DM.

Fecal egg counts and worm studies

In experiment 2, the number of eggs per gram (epg) of feces was estimated using a modified McMaster technique [20] with sodium chloride as the flotation medium, in which every egg is regarded as equivalent to 15 epg fresh feces. In both experiments, the digestive tract of each goat was collected immediately after necropsy, and the small intestine was rapidly opened and washed to recover the parasites. The total number of worms was estimated using a 10% aliquot technique. The rate of larval establishment was calculated according to the results of the worm count as the total number of worms recovered divided by the total number of L3 given, multiplied by 100. To assess nematode development, the length of the female worms was measured using a microscope at x10 magnification. Fertility per female worm was determined via direct counting of the eggs *in utero* after the clearing of each female worm with lactophenol according to the method described by Kloosterman et al. [21] for *Haemonchus contortus*. These procedures were performed on 20 female worms per goat.

Statistical analysis

Data from slaughtered goats (worm burden, female length and fecundity *in utero*) were subjected to one way-analysis of variance (ANOVA) to test the effect of treatments. Log-transformed FEC data from experiment 2 were analysed using a mixed model procedure for repeated measures, with sampling date (11 repeated measures) as the within subject effect on the same animal. All analyses were performed using SAS V8 [22].

Table 1. The mean (S.D.) worm burden (No. of nematodes), establishment rate (%), sex ratio (females/males), female worm length (µm) and fecundity *in utero* (No. of eggs) of *T. colubriformis* in experimentally infected control goats or goats supplemented with heather to test its preventive (experiment 1) and curative (experiment 2) anthelmintic effect.

Experiment 1	Control	Heather		*P*
Worm burden	4,339 (434) a	3,752 (748) a		0.0920
Establishment rate	72.3 (7.2) a	62.5 (12.5) a		0.2569
Sex ratio	0.87 (0.19) a	0.84 (0.22) a		0.7386
Female length	6,112 (712) a	6,397 (605) b		0.0001
Fecundity *in utero*	19.3 (9.8) a	21.6 (8.0) a		0.1188
Experiment 2	**Control**	**Heather**	**Heather + PEG**	***P***
Worm burden	3,408 (1,108) a	2,756 (501) a	3,432 (1,439) a	0.6990
Establishment rate	56.8 (18.5) a	45.9 (8.3) a	57.2 (24.0) a	0.5535
Sex ratio	0.86 (0.23) a	0.66 (0.10) a	0.86 (0.23) a	0.1908
Female length	5,802 (539) a	5,843 (476) a	5,549 (630) b	0.0009
Fecundity *in utero*	22.1 (5.2) a	21.1 (4.7) a	18.9 (5.5) b	0.0002

In experiment 1, 6 controls and 6 supplemented animals were slaughtered at 5 weeks post-infection (pi) (heather was administered 2 weeks before experimental infection and during the infection). In experiment 2, 3 groups of 5 goats (controls, heather-supplemented and heather-supplemented plus PEG) were slaughtered at 11 weeks pi (heather was administered to the supplemented animals from 6 to 11 weeks pi). Means followed by different letters in the same row are significantly different ($P<0.05$).

3. Results

Diets

In both experiments, all offered feed was consumed by each goat, and no refusals were observed. The total phenolic and tannin values in heather were 96 (±7.2) g and 64 (±6.2) g tannic acid equivalents per kg DM, respectively.

Experiment 1: Preventive administration of heather

Heather supplementation was associated with a reduced total nematode count, which was almost significant ($P=0.092$; Table 1). The female nematodes were longer ($P<0.001$) in goats fed with heather (Table 1).

Experiment 2: Curative administration of heather

The goats fed with heather showed a reduction ($P<0.05$) in FEC compared with controls from 45 to 76 days pi, but no differences between the heather and heather plus PEG groups were observed (Figure 1). No differences in the total mean worm burden were found between the groups (Table 1). In the heather plus PEG group, the female *T. colubriformis* length and fecundity *in utero* were lower ($P<0.05$; Table 1)

4. Discussion

The results obtained in the present study suggest that heather consumption by goats at the same time as infection take place caused a reduction in the *T. colubriformis* L3 establishment. Moreover, heather consumption by goats previously infected with *T. colubriformis* and showing a patent infection was associated with a decrease in FEC but was not associated with a reduction in nematode burden.

In the preventive treatment, a tendency of reduction in larval establishment in the heather group compared with the control group was observed. In a similar experiment with goats experimentally infected

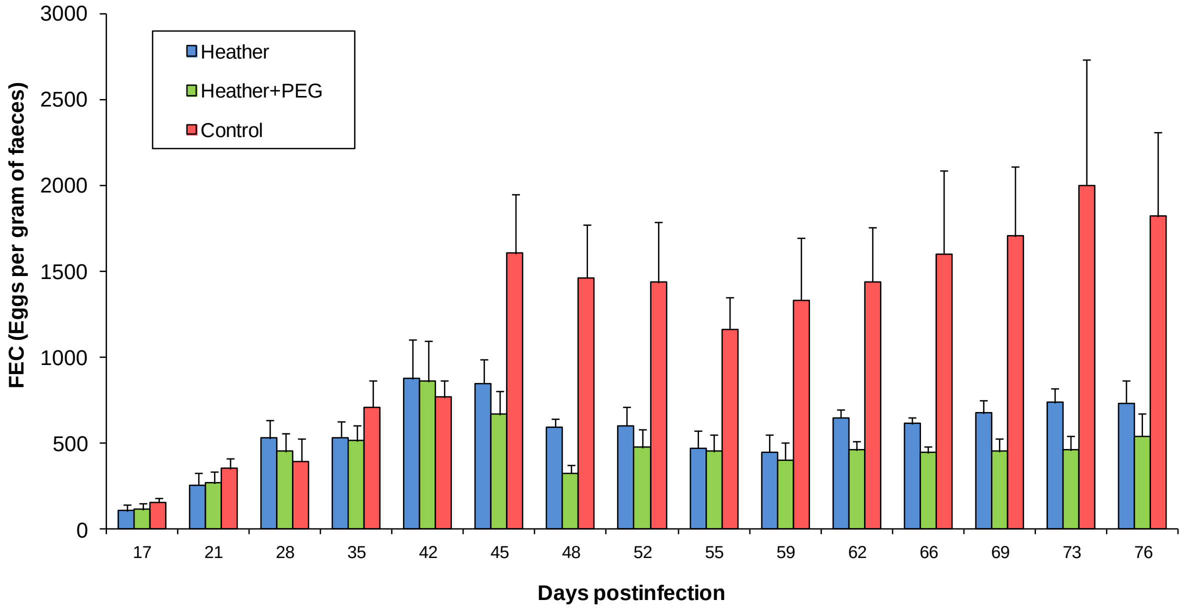

Figure 1. The mean FEC (eggs per gram of fresh faeces) of goats experimentally infected with *T. colubriformis* and supplemented with heather, heather plus polyethylene glycol (PEG) or not supplemented with heather (control). The bars represent the mean and SD. The arrow indicates the beginning of heather administration.

with *T. colubriformis*, Paolini et al. [18] have reported a reduction in female worm fecundity, but no differences in larval establishment were observed when quebracho (a rich condensed tannin supplement extracted from the bark of the tree *Schinopsis* spp.) was administered to animals. However, a decrease in the establishment rate of *T. colubriformis* larvae as early as 5 days pi was observed by Brunet et al. [23] in goats that consumed *Lysiloma latisiliquum* (a tropical tannin-rich tree) leaves. Shaik et al. [14] have also reported a worm burden reduction in goats fed sericea lespedeza (*Lespedeza cuneata*) and naturally infected with *T. colubriformis.* Lambs experimentally infected with *T. colubriformis* and grazing on sulla (*Hedysarum coronarium*) displayed lower worm burdens than those grazing on lucerne [24]. Additionally, in our experiment, worm length and the fecundity levels of female worms seemed to be elevated in the supplemented group. At least in *Teladorsagia circumcincta*, differences in worm fecundity were associated with differences in adult female worm length and the major sources of variation among sheep in worm length appeared to be the immune response and density-dependent constraints on the worm populations [25]. In this case, a higher density of worms in the control goats could explain the shorter length of the adult female worms.

The results of *in vitro* tests with tannin-rich plant extracts suggest a direct effect of these phenolic compounds on different nematode stages, such as *T. colubriformis* L3 [26]. Bahuaud et al. [27] have not reported effects of *Erica erigena* extracts on the *T. colubriformis* L3 exsheathment. Nevertheless, the experimental design used in this experiment (3 weeks between infection time and the slaughter of the goats) did not allow for an assessment of when L3 establishment was exactly disturbed (exsheathment or later processes such as mucosal penetration). Recent *in* *vitro* experiments have shown that heather extracts inhibited or delayed the exsheathment of *T. colubriformis* L3, and the effect was dose-dependent for *C. vulgaris* (Moreno-Gonzalo et al., unpublished data). The anthelmintic effects of heather on the establishment of infective larvae could be explained by the properties of tannins, which interact with the digestive tract of the larvae and/or the surface proteins [28-31]. Other reports including *in vivo* experiments with sheep and goats experimentally and naturally infected have shown differences in the activity levels of the same tannin-rich resource depending on the parasite species and/or their location in the host [13,18,32].

In the curative treatment, heather consumption reduced the mean gastrointestinal nematode egg excretion between 47% and 66% compared with control group 5 weeks after heather administration. This is in agreement with previous field studies performed with goats of the same breed and naturally infected with *T. circumcincta* and *Trichostrongylus* spp. [9,16,17]. Kabasa et al. [33] have observed an FEC increase in naturally infected (*Trichostrongylus* spp. were the most prevalent nematode parasite) goats grazing on *Acacia* spp. plus PEG compared with the control group (without PEG). In another study with goats consuming *Pistacia lentiscus* foliage, the FEC in the tannin plus PEG group was intermediate between the control and treated groups [6]. By studying lambs feeding on cassava (*Manihot esculenta*), Marie-Magdeleine et al. [7] have observed that the mean FEC value for the cassava plus PEG diet group was intermediate between the cassava and lucerne groups (control). PEG administration shows different effects on FEC in lambs feeding on *Acacia cyanophylla* [34,35]. However, in our experiment, FECs in the PEG plus heather group were similar to that of the heather group. PEG seemed to not affect the anthelmintic effect of heather, which could be due to the existence of other compounds in heather that are responsible for the anthelmintic effects or the possibility that the PEG did not completely inhibit the anthelmintic action of the tannins. Niezen et al. [36] have reported that the use of PEG does not seem appropriate in parasitological studies in sheep because there are no consistent results. Finally, the reduction in FEC was observed during the first days of heather consumption; therefore, it is likely that the FEC response could be attributed to a direct effect of the plant secondary compounds on the parasites, and not to an enhanced immune response that could result from an increased protein flow.

In the present study, worm burden tended to be lower in the heather groups than in the control groups; a similar trend was observed for FECs and worm fecundity. As mentioned above for the FEC, PEG utilization does not seem to be very effective, and the results were not clear. PEG seems to increase the humidity level of feces, for this reason FECs expressed as total eggs in faeces per day would be more reliable [34]. Moreover, the effect of PEG could vary from one tannin source to another depending on its different degree of affinity [37]. Therefore, when considering the diversity in tannin bioactivity it is not surprising to observe cases in which PEG failed to neutralize tannin effects on nematodes, as showed in sheep fed with *Lotus pedunculatus* [36].

Currently, it is widely accepted that the concentration of tannins in the diet is a key factor modulating the balance between potential positive and negative tannin-effects. The fact that the treatment including PEG (heather plus PEG) significantly improved the apparent digestibilities of DM (6%) and CP (13%) compared with the control group [38], but both treatments including heather reduced egg excretion levels at the same proportion, might suggest that the threshold of tannins required for the anthelmintic effects is most likely quite low and that the proportion of heather included in the diet could be reduced.

5. Conclusion

The overall results suggest that heather consumption reduces incoming larvae establishment if it is consumed during the infection and reduces FEC by means of a combination between worm burden and female worm fecundity reduction if it is consumed when goats are suffering a patent infection of *T. colubriformis*.

Abbreviations

CP: Crude protein; DM: Dry matter; epg: Eggs per gram; FEC: Fecal egg counts; L3: Third stage larvae; PEG: Polyethylene glycol; pi: Post-infection; BW: Body weight.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors contributed equally to this work. JMG carried out the parasitological studies (fecal egg counts and worm studies) and drafted the manuscript. UG performed the field work. PF, RC and LMMF analyzed heather and diet composition and drafted the manuscript. KO, LMOM and IF participated in study design and coordination, and drafted the manuscript. All authors read and approved the manuscript.

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References

1. Hoste H, Jackson F, Athanasiadou S, Thamsborg SM, Hoskin SO: **The effects of tannin-rich plants on parasitic nematodes in ruminants**. Trends Parasitol 2006, **22**:253–261.

2. Waller PJ, Thamsborg SM: **Nematode control in "green" ruminant production systems**. Trends Parasitol 2004, **20**:493-497.

3. Min BR, Barry TN, Attwood GT, McNabb WC: **The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: a review**. Anim. Feed Sci Technol 2003, **106**:3-19.

4. Celaya R, Ferreira LMM, Moreno-Gonzalo J, Frutos P, Hervás G, Ferre I, García U, Ortega-Mora LM, Osoro K: **Effects of heather and oat supplementation on gastrointestinal nematode infections and performance of grazing Cashmere goats**. Small Rumin Res 2010, **91**:186-192.

5. Hussain A, Khan MN, Iqbal Z, Sajid MS, Khan MK: **Anthelmintic activity of *Trianthema portulacastrum* L. and *Musa paradisiaca* L. against gastrointestinal nematodes of sheep**. Vet Parasitol 2011, **179**:92-99.

6. Landau S, Azaizeh H, Muklada H, Glasser T, Ungar ED, Baram H, Abbas N, Markovics A: **Anthelmintic activity of *Pistacia lentiscus* foliage in two Middle Eastern breeds of goats differing in their propensity to consume tannin-rich browse**. Vet Parasitol 2010, **173**:280-286.

7. Marie-Magdeleine C, Mahieu M, Philibert L, Despois P, Archimède H: **Effect of cassava (*Manihot esculenta*) foliage on nutrition, parasite infection and growth of lambs**. Small Rumin Res 2010, **93**:10-18.

8. Moreno FC, Gordon IJ, Knox MR, Summer PM, Skerrat LF, Benvenutti MA, Saumell CA: **Anthelmintic efficacy of five tropical native Australian plants against *Haemonchus contortus* and *Trichostrongylus colubriformis* in experimentally infected goats (*Capra hircus*)**. Vet Parasitol 2012, **187**:237-243.

9. Osoro K, Celaya R, Moreno-Gonzalo J, Ferreira LMM, García U, Frutos P, Ortega-Mora LM, Ferre I: **Effects of stocking rate and heather supplementation on gastrointestinal nematode infections and host performance in naturally infected Cashmere goats**. Rangeland Ecol Manage 2009, **62**:127-135.

10. Terrill TH, Dykes GS, Shaik SA, Miller JE, Kouakou B, Kannan G, Burke JM, Mosjidis JA: **Efficacy of sericea lespedeza hay as a natural dewormer in goats: Dose titration study**. Vet Parasitol 2009, **163**:52-56.

11. Min BR, Hart SP, Miller D, Tomita GM, Loetz E, Sahlu T: **The effect of grazing forage containing condensed tannins on gastro-intestinal parasite infection and milk composition in Angora does**. Vet Parasitol 2005, **130**:105-113.

12. Moreno-Gonzalo J, Ferre I, Celaya R, Frutos P, Ferreira LMM, Hervás G, García U, Ortega-Mora LM, Osoro K: **Potential use of heather to control gastrointestinal nematodes in goats**. Small Rumin Res 2012, **103**:60–68.

13. Paolini V, De La Farge F, Prevot F, Dorchies P, Hoste H: **Effects of the repeated distribution of sainfoin hay on the resistance and the resilience of goats naturally infected with gastrointestinal nematodes.** Vet Parasitol 2005, **127**:277-283.

14. Shaik SA, Terrill TH, Millar JE, Kouakou B, Kannan G, Kaplan RM, Burke JM, Mosjidis, JA: **Sericea lespedeza hay as a natural deworming agent against gastrointestinal**

nematode infections in goats. Vet Parasitol 2006, **139**:150-157.

15. Frutos P, Moreno-Gonzalo J, Hervás G, García U, Ferreira LMM, Celaya R, Toral PG, Ortega-Mora LM, Ferre I, Osoro K: **Is the anthelmintic effect of heather supplementation to grazing goats always accompanied by anti-nutritional effects?** Animal 2008, **2**:1449-1456.

16. Osoro K, Benito-Peña A, Frutos P, García U, Ortega-Mora L.M, Celaya R, Ferre I: **The effect of heather supplementation on gastrointestinal nematode infections and performance in Cashmere and local Celtiberic goats on pasture**. Small Rumin Res 2007, **67**:184-191.

17. Osoro K, Mateos-Sanz A, Frutos P, García U, Ortega-Mora LM, Ferreira LMM, Celaya R, Ferre I: **Anthelmintic and nutritional effects of heather supplementation on Cashmere goats grazing perennial ryegrass-white clover pastures**. J. Anim. Sci. 2007, **85**:861-870.

18. Paolini V, Frayssines A, De La Farge F, Dorchies P, Hoste H: **Effects of condensed tannins on established populations and on incoming larvae of *Trichostrongylus colubriformis* and *Teladorsagia circumcincta* in goats**. Vet Res 2003, 34:331-339.

19. Makkar HPS, Blummel M, Borowy NK, Becker K: **Gravimetric determination of tannins and their correlations with chemical and protein precipitation method**. J Sci Food Agric 1993, **61**:161-165.

20. [MAFF] Ministry of Agriculture, Fisheries, and Food: Manual of veterinary parasitological techniques, 3rd ed. Reference Book 418, MAFF, London; 1986.

21. Kloosterman A, Albers GAA, van der Brink H: **Genetic variations among calves in resistance to nematode parasites**. Vet Parasitol 1978, **4**:353-368.

22. SAS: SAS/STAT User´s Guide. V8. SAS Institute Inc., Cary, NC, USA; 1999.

23. Brunet S, Martinez-Ortiz de Montellano C, Torres-Acosta JFJ, Sandoval-Castro CA, Aguilar-Caballero AJ, Capetillo-Leal C, Hoste H: **Effect of the consumption of *Lysiloma latisiliquum* on the larval establishment of gastrointestinal nematodes in goats**. Vet Parasitol 2008, **157**:81-88.

24. Niezen JH, Waghorn TS, Charleston WAG, Waghorn GC: **Growth and gastrointestinal nematode parasitism in lambs grazing lucerne (*Medicago sativa*) or sulla (*Hedysarum coronarium*) which contain condensed tannins**. J Agric Sci Camb 1995, **125**:281-289.

25. Stear MJ, Bishop SC**: The curvilinear relationship between worm length and fecundity of *Teladorsagia circumcincta***. Int J Parasitol 1999, **29**:777-780.

26. Alonso-Diaz MA, Torres-Acosta JF, Sandoval-Castro CA, Capetillo-Leal C, Brunet S, Hoste H: **Effects of four tropical tanniferous plant extracts on the inhibition of larval migration and the exsheathment process of *Trichostrongylus colubriformis* infective stage**. Vet Parasitol 2008, **153**:187-192.

27. Bahuaud D, Martinez-Ortiz De-Montellano C, Chauveau S, Prevot F, Torres-Acosta F, Fouraste I, Hoste H: **Effects of four tanniferous plant extracts on the *in vitro* exsheathment of third-stage larvae of parasitic nematodes**. Parasitology 2006, **132**:545-554.

28. Molan AL, Meagher LP, Spencer PA, Sivakumaran S: **Effect of flavan-3-ols on *in vitro* egg hatching, larval development and viability of infective larvae of *Trichostrongylus colubriformis***. Int J Parasitol 2003, **33**:1691-1698.

29. Molan AL, Sivakumaran S, Spencer, PA, Meagher LP: **Green tea flavan-3-ols and oligomeric proanthocyanidins inhibit the motility of infective larvae of *Teladorsagia circumcincta* and *Trichostrongylus colubriformis in vitro***. Res Vet Sci 2004, **77**:239-243.

30. Brunet S, Aufrere J, El Babili F, Fouraste I, Hoste H: **The kinetics of exsheathment of infective nematode larvae is disturbed in the presence of a tannin-rich plant extract (sainfoin) both *in vitro* and *in vivo***. Parasitology 2007, **134**:1253-1262.

31. Brunet S, Jackson F, Hoste H,**. Effects of sainfoin (*Onobrychis viciifolia*) extract and monomers of condensed tannins on the association of abomasal nematode larvae with fundic explants**. Int J Parasitol 2008, **38**:783-790.

32. Athanasiadou S, Kyriazakis I, Jackson F, Coop RL: **Direct anthelmintic effects of condensed tannins towards different gastrointestinal nematodes of sheep: *in vitro* and *in vivo* studies**. Vet Parasitol 2001, **99**:205-219.

33. Kabasa JD, Opuda-Asibo J, ter Meulen U: **The effect of oral administration of polyethylene glycol on faecal helminth egg counts in pregnant goats grazed on browse containing condensed tannins**. Trop Anim Health Prod 2000, **32**:73-86.

34. Akkari H, Ben Salem H, Gharbi M, Abidi S, Darghouth MA: **Feeding *Acacia cyanophylla* Lindl. foliage to Barbarine lambs with or without PEG: Effect on the excretion of gastro-intestinal nematode eggs**. Anim Feed Sci Technol 2008, **147**:182-192.

35. Akkari H, Darghouth MA, Ben Salem H: **Preliminary investigations of the anti-nematode activity of *Acacia cyanophylla* Lindl.: Excretion of gastrointestinal nematode eggs in lambs browsing *A. cyanophylla* with and without PEG or grazing native grass**. Small Rumin Res 2008, **74**:78-83.

36. Niezen JH, Waghorn GC, Charleston WAG: **Establishment and fecundity of *Ostertagia circumcincta* and *Trichostrongylus colubriformis* in lambs fed lotus (*Lotus pedunculatus*) or perennial ryegrass (*Lolium perenne*)**. Vet Parasitol 1998, **78**:13-21.

37. Ben Salem H, Nefzaoui A, Ben Salem L, Tisserand JL: **Intake, digestibility, urinary excretion of purine derivatives and growth by sheep given fresh, air-dried or polyethylene glycol-treated foliage of *Acacia cyanophylla* Lindl**. Anim Feed Sci Technol, 1999, **78**:297-311.

38. Frutos P, Hervás G, Celaya R, Moreno-Gonzalo J, Rodríguez AB, García U, Ferre I, Ortega-Mora LM, Osoro K: **Effects of the use of heather as anthelmintic in goats infected with *Trichostrongylus colubriformis* on ruminal fermentation and digestibility**. Opt Méd 2011, **99**:321-327.

Effect of the consumption of heather on incoming larvae and established population of *Teladorsagia circumcincta* in experimentally infected Cashmere goats

J. Moreno-Gonzalo [a], K. Osoro [b], U. García [b], P. Frutos [c], R. Celaya [b], L.M.M. Ferreira [d], L.M. Ortega-Mora [a], I. Ferre [a,*]

[a] *SALUVET Group, Department of Animal Health, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040-Madrid, Spain*

[b] *Servicio Regional de Investigación y Desarrollo Agroalimentario (SERIDA), Área de Sistemas de Producción Animal, 33300-Villaviciosa, Asturias, Spain*

[c] *Instituto de Ganadería de Montaña, CSIC-ULE, Finca Marzanas, 24346-León, Spain*

[d] *CECAV, Departamento de Zootecnia, Universidade de Trás-os-Montes e Alto Douro, PO Box 1013, 5000-911 Vila Real, Portugal*

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Summary

This study was performed in Cashmere goats that were experimentally infected with *Teladorsagia circumcincta* to investigate the effects of heather consumption on the establishment of incoming infective larvae (experiment 1) and on an adult nematode population (experiment 2). In experiment 1, 24 non-lactating goats were divided into 2 groups: heather-supplemented *vs.* non-supplemented. After 2 weeks of adaptation to the diet, all of the goats were experimentally infected with 6,000 infective larvae of *T. circumcincta*. Twelve animals (6 controls and 6 supplemented with heather) were slaughtered at 6 days and at 3 weeks post-infection (pi). After slaughter, the worms were counted and the female worm fecundity and development were determined. Heather consumption was associated with a significant reduction in larval establishment at 6 days (P=0.033) and at 3 weeks (P=0.041) pi. No differences in worm counts between the slaughter times were found. In the goats slaughtered at 3 weeks pi, the number of eggs *in utero* and length of the female worms were significantly (P<0.001) lower than those of control group. In experiment 2, 24 non-lactating goats were experimentally infected with 10,000 *T. circumcincta* infective larvae daily for 5 consecutive days (total infection of 50,000 larvae). After 3 weeks, 2 groups were established: control and heather-supplemented. The faecal egg output of each animal was measured at 2-days interval during the experimental period. The goats were slaughtered after 3 weeks of heather administration for parasitological studies. Heather administration was associated with a significant decrease in egg excretion between 25 and 29 days pi. The worm counts were similar in both groups, but the female length and fecundity were significantly (P<0.001) lower in supplemented goats. These results show that heather consumption reduces the establishment of *T. circumcincta* larvae in goats and the development and fecundity of female adult parasites.

Keywords: *Teladorsagia circumcincta*; Goat; Heather; Tannin; Control

1. Introduction

An increasing number of studies have shown that the consumption of bioactive plants containing secondary metabolites, such as tannins, by small ruminants can regulate their gastrointestinal (GI) nematode population (Brunet et al., 2008b; Terrill et al., 2009; Manolaraki et al., 2010), thus reducing their dependence on conventional chemotherapy and supporting the possibility of a sustainable mechanism to control GI parasitism (Waller and Thamsborg, 2004; Hoste et al., 2006; Waller, 2006).

Heather species belong to the Ericaceae family (*Erica* spp. or *Calluna vulgaris*) have a moderate to high tannin content (ranging from 30 to 100 g tannic acid equivalents per kg dry matter (DM), Moreno-Gonzalo et al., 2012) but low nutritive value (Frutos et al., 2002). Heather is a common component of the natural vegetation found in mountain communities in humid temperate areas. Goat diets include high proportions of heather because goats browse on heathlands even when pastures are available (Celaya et al., 2007). Recently, a number of studies have been conducted to investigate the anthelmintic and nutritional effects of heather supplementation on goats naturally infected by GI nematodes (Osoro et al., 2007a, b, 2009; Frutos et al., 2008; Celaya et al., 2010). The results of these studies suggest that (i) heather supplementation significantly reduces the level of GI nematode egg excretion in grazing goats, (ii) the faecal nematode egg counts (FEC) reduction could be associated with a decrease in worm fertility and/or a reduction in the establishment of incoming third-stage larvae, (iii) the consumption of heather is associated with an apparent increase in the resilience of the goats to GI nematode infections, and (iv) the amount of tannins consumed by goats offered heather does not seem to be associated with substantial anti-nutritional effects, which eventually resulted in a better performance in the animals incorporating these shrubs in their diet.

A number of field experiments performed with grazing goats have shown that many plants have anthelmintic activity (Kabasa et al., 2000; Min et al., 2004, 2005; Pomroy and Adlington, 2006; Moore et al., 2008; Landau et al., 2010), including heather (Moreno-Gonzalo et al., 2012). However, grazing experiments with goats that harbour mixed GI nematode infections are strongly dependent on climatic conditions, and the number and the development stages of the nematodes present in the GI tract cannot be precisely determined by FEC and coprocultures. Additionally, the effects of the consumption of tannin-containing plants could vary depending on the stage of the parasites upon exposure to the tannins (Paolini et al., 2003c). *Teladorsagia circumcincta* is the most prevalent gastric nematode found in goats in temperate dry and humid areas, such as Central and Northwest Spain (Valcárcel and Romero, 1999; Álvarez-Sánchez et al., 2006). The present study was performed in Cashmere goats experimentally infected with *T. circumcincta* to investigate the effects of heather consumption on the establishment of incoming infective larvae (experiment 1: preventive administration), and on the adult parasite population (experiment 2: curative administration).

2. Materials and methods

2.1. Experimental site

The experiments were conducted at the "El Carbayal" research station in northwest Spain (6° 53´ W, 43° 20´ N, Sierra de San Isidro, Illano, Asturias) in accordance with the Spanish Royal Decree 1201/2005 (based on European Council Directive 86/609/ECC) for the protection of animals used for experimental and other scientific purposes.

2.2. Experimental designs

2.2.1. Experiment 1: Preventive administration of heather

Twenty-four, 30-50 months old, non-lactating Cashmere does showing <100 trichostrongylid eggs per gram (epg) of faeces were dewormed with ivermectin (Oramec, Merial, Lyon, France) 4 weeks before the experiment start date. The trial lasted for 5 weeks and was divided into 2 successive periods: a 2-weeks period for adaptation to the diet and a 3-weeks experimental period. The goats (showing no nematode eggs in coproscopic examination) were divided into 2 groups of 12 animals each, balanced according to their body weight (32.7±1.85 kg BW): heather supplemented (70% alfalfa hay + 30% heather) *vs*. non-supplemented (alfalfa hay). Diets (30 g DM kg $BW^{-0.75}$ day^{-1}) were offered twice a day (at 9:00 h and 18:00 h), accounting for an average of 410 g DM $goat^{-1}$ day^{-1} as calculated for the goats mean BW. The amounts were below the maintenance requirements of non-lactating, non-pregnant goats and were conceived to avoid refusals. Heather was cut every 3 days in a nearby heathland area belonging to the experimental farm and stored chilled until it was supplied to the animals. *Calluna vulgaris* accounted for more than 90% of the supplied heather, with scarce amounts of *Erica umbellata* and *Erica cinerea*. Total phenolic and tannin values in heather were 76.5 (±4.9) and 48.2 (±2.3) g tannic acid equivalents per kg DM respectively (assays for heather total phenolics and tannins were conducted following the Folin-Ciocalteu technique in combination with polyvinyl-polypyrrolidone, using tannic acid as the reference standard, Makkar et al., 1993). After 2 weeks of adaptation to the diets, all of the goats were experimentally infected with 6,000 *T. circumcincta* infective larvae (supplied by the Moredun Research Institute, Scotland, UK). During the experimental period, the goats were maintained in individual metabolic cages with unrestricted water, and their food intake was monitored. Twelve animals (6 controls and 6 supplemented with heather) were slaughtered at 6 days post-infection (pi) and the other 12 at 3 weeks pi (3 and 5 weeks, respectively, after heather administration in the supplemented group). After slaughter, the worms were counted and the female worm fecundity and development were determined.

2.2.2. Experiment 2: Curative administration of heather

Twenty-four, 30-60 months old, non-lactating Cashmere does showing <150 trichostrongylid epg of faeces were dewormed with ivermectin (Oramec, Merial, Lyon, France) 4 weeks before the experiment began. The trial lasted for 6 weeks and was divided into 2 successive periods: a 3-weeks period for the establishment of an adult nematode population and a 3-weeks experimental period including the administration of heather in the supplemented group. All of the does (showing no trichostrongylid eggs in coproscopic examination) were experimentally infected by a trickle infection (10,000 *T. circumcincta* infective larvae daily) for 5 consecutive days (total infection 50,000 larvae). The infective larvae were supplied by the Moredun Research Institute (Scotland, UK). The goats were maintained in a pen with a concrete floor and fed alfalfa hay *ad libitum* from anthelmintic treatment to 3 weeks pi. Three weeks after infection, 2 groups of goats (heather-supplemented *vs*. non-supplemented) were established according to BW and the level of nematode egg excretion. Goats were placed in individual metabolic cages to monitor food consumption, and water was provided *ad libitum*. The diets were offered as in experiment 1. Total phenolic and tannin values in heather were 80.3 (±2.1) and 51.6 (±9.8) g tannic acid equivalents per kg DM respectively. The individual faecal egg output was measured at 2-days interval during the experimental period. The goats were slaughtered after 6 weeks pi (3 weeks after heather administration in supplemented group) for parasitological studies.

2.3. Parasitological procedures

In experiment 2, individual faecal samples were collected from the rectum to measure nematode egg excretion. The number of *T. circumcincta* epg of faeces was estimated using the modified McMaster technique (MAFF, 1978) with sodium chloride as the flotation medium in which every egg is regarded as equivalent to 15 epg of fresh faeces.

In both experiments, the digestive tracts were collected immediately (no more than 10 minutes) after slaughter and the abomasa were rapidly opened and washed to recover the parasites. The total numbers of worms were estimated from a 10% aliquot technique. The rates of larval establishment were calculated according to the results of the worm counting as the total number of worms recovered divided by the total number of infective larvae given and multiplied by 100. To assess the nematode development, the lengths of the female worms were measured using a microscope at x10 magnification. The fertility per female worm was determined by direct counting of the eggs *in utero* after clearing each female worm with lactophenol according to the method described by Kloosterman et al. (1978) for *Haemonchus contortus*. These procedures were performed on at least 20 female worms per goat.

2.4. Statistical analysis

The data from the slaughtered goats (worm burden, female length and fecundity *in utero*) were subjected to one way-analysis of variance (ANOVA) tests to examine the effects of the treatments. The log-transformed FEC data from experiment 2 were analysed using a mixed model procedure for repeated measures, with the sampling date (11 repeated measures at 2-days interval) as the within-subject effect on the same animal. All of the analyses were performed using SAS V8 (SAS Institute Inc., Cary, NC). Differences were considered significant when $P<0.05$.

3. Results

In both experiments, all offered food was consumed daily without refusals.

3.1. Experiment 1: Preventive administration of heather

Heather consumption was associated with a significant reduction in larval establishment at 6 days pi ($P=0.033$) and a significant reduction in the nematode counts at 3 weeks pi ($P=0.041$) (Table 1). No differences in worm counts between the slaughter times were found. In goats slaughtered at 3 weeks pi, the number of eggs *in*

Table 1. Mean (S.E.) worm burden (No. of nematodes), establishment rate (%), female worm length (µm), and fecundity *in utero* (No. of eggs) of *Teladorsagia circumcincta* in experimentally infected control goats or goats supplemented with heather. In the preventive experiment, groups of 12 (6 controls and 6 supplemented) animals were slaughtered at 6 days post-infection (pi) and at 3 weeks pi (heather was administered 2 weeks before experimental infection and during the infection). In the curative experiment, the goats (12 controls and 12 supplemented) were slaughtered at 6 weeks pi (heather was administered to supplemented animals from 3 to 6 weeks pi).

Experiment	Control	Heather	P
Preventive (6 days pi)			
Worm burden	2,475 (468)	1,450 (170)	0.033
Establishment rate *	41.3 (7.8)	24.2 (2.8)	
Preventive (3 weeks pi)			
Worm burden	2,502 (363)	1,375 (216)	0.041
Establishment rate *	41.7 (8.2)	22.9 (5.0)	
Female length	10,369 (125)	10,038 (99)	0.0001
Fecundity *in utero*	36 (1.8)	25 (1.5)	0.0001
Curative			
Worm burden	3,829 (557)	3,868 (632)	0.888
Establishment rate *	7.7 (1.2)	7.7 (1.1)	
Female length	10,068 (65)	9,850 (87)	0.0001
Fecundity *in utero*	28 (1.1)	22 (1.0)	0.0001

* Establishment rate in control group was considered 100% for calculation of reduction of establishment of worms in heather-supplemented group.

utero was lower (P<0.001) and the length of the female worms was shorter (P<0.001) from the heather-supplemented animals than those recorded in the control group (Table 1).

3.2. Experiment 2: Curative administration of heather

Heather administration was associated with a decrease in egg excretion between 25 and 29 days pi (Figure 1). Between 31 and 41 days pi, the egg output of heather-supplemented animals was slightly lower than that of the controls (Figure 1). The worm counts were similar in both groups, but the length and fecundity of the female *T. circumcincta* were significantly (P<0.001) lower in the supplemented goats (Table 1).

4. Discussion

A number of controlled *in vivo* experiments have shown the anthelmintic effect of tannin-containing plants that are common to temperate areas such as sainfoin (*Onobrychis viciifolia*, Paolini et al., 2003b, 2005; Hoste et al., 2005), sericea lespedeza (*Lespedeza cuneata*, Min et al., 2004, 2005; Shaik et al., 2006; Terrill et al., 2007, 2009; Moore et al., 2008), sulla (*Hedysarum coronarium*, Pomroy and Adlington, 2006), and *Viscum verrocosum* (Madibela and Jansen, 2003) in goats, but none of these studies have assessed the effect of heather species. The present study was performed to investigate the effect of heather supplementation on incoming infective larvae and adult parasites in goats experimentally

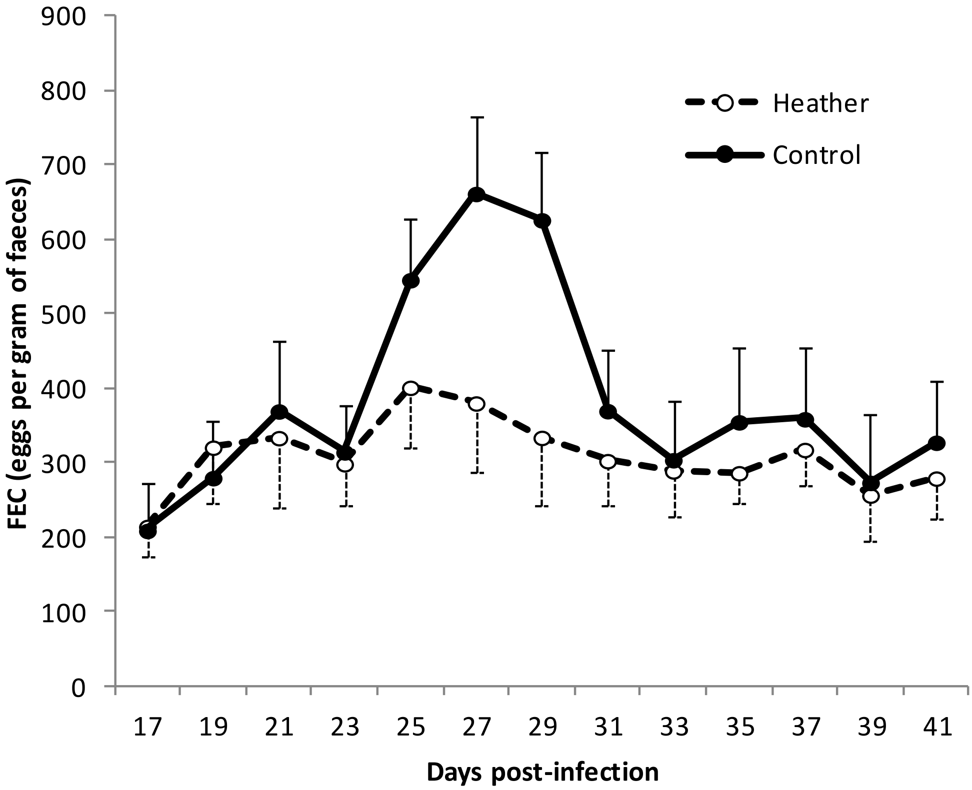

Figure 1. Mean (S.E.) FEC (eggs per gram of faeces) of goats (12 animals per group) that were experimentally infected with *Teladorsagia circumcincta* and supplemented with heather (broken line) or fed alfalfa hay-only diet (solid line) to test the curative effect of heather against gastrointestinal nematodes. The arrow represents the beginning of the heather administration (at 21 days post-infection).

infected with *T. circumcincta*. The effects of tannin-containing plants on GI nematodes are dependent on the parasite species, parasitic stage and the plant species (Hoste et al., 2006). In a previous study on Cashmere goats that grazed on upland perennial ryegrass (*Lolium perenne*)-white clover (*Trifolium repens*) pastures, a reduction in the adult *T. circumcincta* counts was reported in animals that were supplemented with heather (Osoro et al., 2009). However, the effect of the heather on the worm population was measured after several weeks of infection, and it was not possible to distinguish any specific effect on the establishment of the larvae. Necropsies were carried out at the end of the experiment; thus it was not possible to determine whether the differences in the worm population occurred at the beginning or end of the establishment process or if the worm burden reduction was caused by deaths in the adult worm population. Additionally, the possibility that the heather-supplemented goats consumed fewer infective larvae due to appetite depression should be considered as an alternative to a direct effect of the heather on the worm population (Coop and Kyriazakis, 1999; Hoste et al., 2008). In the present study, the level of infection and the amount of heather consumed by each goat was controlled to avoid external factors that could hamper the interpretation of results.

The main result of the preventive administration of heather was a decrease in larval establishment in the heather-supplemented goats. This reduction was observed as early as 6 days after infection and represented an overall 43% decrease when compared to non-supplemented animals. The worm counts were similar at 3-weeks pi, suggesting that the dominant effect of heather was during the exsheathment process or at the beginning of parasite establishment (mucosal penetration). Paolini et al. (2003c) also observed a reduction in *Trichostrongylus colubriformis* and *T. circumcincta* burdens at 3-weeks pi in goats fed quebracho (a rich condensed tannin supplement extracted from the bark of the tree *Schinopsis* spp.). Others have shown that feeding goats with *Lysiloma latisiliquum* leaves resulted in the reduction of *H. contortus* and *T. colubriformis* infective larval establishment at 5 days pi (Brunet et al., 2008b). However, lambs that grazed on chicory *(Cichorium intybus*, although its anthelmintic activity is probably related to sesquiterpene lactones instead of condended tannins*)* or sulla showed no significant differences in immature *T. circumcincta* counts at 7 days after their experimental infection (Tzamaloukas et al., 2005).

Our results suggest that heather has a direct effect on the exsheathment process or the first stage of larval establishment. Brunet et al. (2008a) reported a reduction in the penetration of exsheathed larvae of *T. circumcincta* into abomasal fundic explants after incubation with a sainfoin extract. Bahuaud et al. (2006) showed that the exsheatment of *H. contortus* infective larvae was significantly delayed *in vitro* when incubated with heather extracts from *Erica erigena*. However, no differences were found when *T. colubriformis* infective larvae were incubated with the same extracts. Further *in vitro* studies are needed to clarify the real effect of heather compounds on *T. circumcincta* larvae.

Another notable observation from the preventive administration of heather was that female fecundity was lower in nematodes isolated at 3-weeks pi from animals supplemented with heather. Therefore, heather could have had an effect on the worms after the exsheathment process that did not induce the death of parasites but rather affected their development and fecundity as the length measurements suggest. Brunet et al. (2011) observed ultrastructural changes in the third-stage infective nematode larvae (both exsheathed and ensheathed) by the action of sainfoin extracts. Furthermore, an indirect mechanism has also been suspected because tannins protect proteins against ruminal degradation (Min et al., 2003) and their consumption could stimulate the host immune response (Coop and Kyriazakis, 1999). The immune response includes exclusion of incoming infective larvae, slowing of larval development, and effects on adult worm length and fecundity (Mcneilly et al., 2009). Experiments with lambs have shown a strong relationship between decreased worm length and decreased worm fecundity. The resistance in lambs appears to be due to control of worm growth and not to control of worm numbers. The only immune response that is consistently associated with reduced worm length is the response to fourth-stage larvae of *T. circumcincta* (Stear et al., 1999).

Studies performed in sheep (Niezen et al., 1998; Athanasiadou et al., 2001, Marie-Magdeleine et al., 2010; Mupeyo et al., 2011) and goats (Paolini et al., 2003a, b, 2005; Min et al., 2005, Sokerya et al., 2009) showed a decrease in the FEC of animals that consumed tannin-containing plants, forages or their extracts. In previous experiments with grazing goats supplemented with heather (Osoro et al., 2007a, b; Celaya et al., 2010) a significant decrease in the FEC was also observed. However, no necropsies were performed, and it was not possible to associate the reduction in FEC with a lower nematode burden or decreased worm fecundity. The curative experiment was performed to investigate the effect of heather consumption on an established population of *T. circumcincta* in experimentally infected goats and to assess whether the FEC decrease was associated with a lower worm burden or with a decrease in fecundity of female worms (or both). A reduction in nematode egg excretion was observed as early as 5 days after heather administration, suggesting that heather has a direct effect on worms. The decrease in egg output observed when tannin-containing heather was consumed by goats infected with an established population of *T. circumcinta* was not due to a reduction in worm counts but was instead associated with reduced fecundity in female worm. These observations agree with results reported by Paolini et al. (2003c) and Ríos-de Álvarez et al. (2012) from their studies on the effects of quebracho or lectins (extracted from *Phaseolus vulgaris*) administered to goats and sheep, respectively, that were infected with *T. circumcincta*. Other studies on sheep experimentally infected with the same abomasal nematode and fed different condensed tannin sources found a decrease in the worm burden associated (Niezen et al., 1998; Mupeyo et al.,2011) or not associated (Niezen et al., 2002; Tzamaloukas et al., 2005) with a decrease in female worm fecundity. Athanasiadou et al. (2001) found no differences in either the worm burden or the FEC in sheep that were fed quebracho and experimentally infected with *T. circumcincta*. Our results suggest that heather did not affect the *T. circumcincta* worm number after the parasites were established. However, heather supplementation led to a reduction in egg excretion that could be associated with decreased female worm fecundity. However these effects could be reversible. The heather was administered continuously from 3 weeks pi and the effects seem to be higher in the period of maximum *T. circumcincta* egg output (3-5 weeks pi) tending to be lower afterwards.

The overall results showed that heather consumption could restrict the establishment of incoming larvae (lower infection) and the egg excretion of adult female worms of *T. circumcincta* (lower pasture contamination). These observations are in accordance with previously reported grazing trials (Moreno et al., 2012). Heather supplementation in goats at pasture could be a useful alternative or a complementary method for GI nematode control. However, additional research and *in vitro* studies are needed to verify the direct effect of heather (and the role of tannins) on the parasites.

Conflict of interest

None.

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References

Álvarez-Sánchez, M., Pérez-García, J., Cruz-Rojo, M., Rojo-Vázquez, F., 2006. Anthelmintic resistance in trichostrongylid nematodes of sheep farms in Northwest Spain. Parasitol. Res. 99, 78-83.

Athanasiadou, S., Kyriazakis, I., Jackson, F., Coop, R.L., 2001. Direct anthelmintic effects of condensed tannins towards different gastrointestinal nematodes of sheep: *in vitro* and *in vivo* studies. Vet. Parasitol. 99, 205-219.

Bahuaud, D., Martínez-Ortíz de Montellano, C., Chauveau, S., Prevot, F., Torres-Acosta, F., Fouraste, I., Hoste, H., 2006. Effects of four tanniferous plant extracts on the *in vitro* exsheatment of third-stage larvae of parasitic nematodes. Parasitology 132, 545-554.

Brunet, S., Jackson, F., Hoste, H., 2008a. Effects of sainfoin (*Onobrychis viciifolia*) extract and monomers of condensed tannins on the association of abomasal nematode larvae with fundic explants. Int. J. Parasitol. 38, 783-790.

Brunet, S., Martinez-Ortiz de Montellano, C., Torres-Acosta, J.F.J., Sandoval-Castro, C.A., Aguilar-Caballero, A.J., Capetillo-Leal, C., Hoste, H., 2008b. Effect of the consumption of *Lysiloma latisiliquum* on the larval establishment of gastrointestinal nematodes in goats. Vet. Parasitol. 157, 81-88.

Brunet, S., Fourquaux I., Hoste H., 2011. Ultrastructural changes in the third-stage, infective larvae of ruminant nematodes treated with sainfoin (*Onobrychis viciifolia*) extract. Parasitol. Int. 60, 419–424.

Celaya, R., Oliván, M., Ferreira, L.M.M., Martínez, A., García, U., Osoro, K., 2007. Comparison of grazing behaviour, dietary overlap and performance in non-lactating domestic ruminants grazing on marginal heathland areas. Livest. Sci. 106, 271-281.

Celaya, R., Ferreira, L.M.M., Moreno-Gonzalo, J., Frutos, P., Hervás, G., Ferre, I., García, U., Ortega-Mora, L.M., Osoro, K., 2010. Effects of heather and oat supplementation on gastrointestinal nematode infections and performance of grazing Cashmere goats. Small Rumin. Res. 91, 186-192.

Coop, R.L., Kyriazakis, I., 1999. Nutrition-parasite interaction. Vet. Parasitol. 84, 187-204.

Frutos, P., Hervás, G., Ramos, G., Giráldez, F.J., Mantecón, A.R., 2002. Condensed tannin content of several shrub species from a mountain area in northern Spain and its relationship to various indicators of nutritive value. Anim. Feed Sci. Technol. 95, 215-226.

Frutos, P., Moreno-Gonzalo, J., Hervás, G., García, U., Ferreira, L.M.M., Celaya, R., Toral, P.G., Ortega-Mora, L.M., Ferre, I., Osoro, K., 2008. Is the anthelmintic effect of heather supplementation to grazing goats always accompanied by anti-nutritional effects? Animal 2, 1449-1456.

Hoste, H., Gaillard, L., Le Frileux, Y., 2005. Consequences of the regular distribution of sainfoin hay on gastrointestinal parasitism with nematodes and milk production in dairy goats. Small Rumin. Res. 59, 265-271.

Hoste, H., Jackson, F., Athanasiadou, S., Thamsborg, S.M., Hoskin, S.O., 2006. The effects of tannin-rich plants on parasitic nematodes in ruminants. Trends Parasitol. 22, 253-261.

Hoste, H., Torres-Acosta, J.F.J., Aguilar-Caballero, A.J., 2008. Nutrition-parasite interactions in goats: is immunoregulation involved in the control of gastrointestinal nematodes? Parasite Immunol. 30, 79-88.

Kabasa, J.D., Opuda-Asibo, J., ter Meulen, U., 2000. The effect of oral administration of polyethylene glycol on faecal helminth egg counts in pregnant goats grazed on browse containing condensed tannins. Trop. Anim. Health Prod., 32, 73-86.

Kloosterman, A., Albers, G.A.A., van der Brink, H., 1978. Genetic variations among calves in resistance to nematode parasites. Vet. Parasitol. 4, 353-368.

Landau, S., Azaizeh, H., Muklada, H., Glasser, T., Ungar, E.D., Baram, H., Abbas, N., Markovics, A., 2010. Anthelmintic activity of *Pistacia lentiscus* foliage in two Middle Eastern breeds of goats differing in their propensity to consume tannin-rich browse. Vet. Parasitol., 173, 280-286.

Madibela, O.R., Jansen, K., 2003. The use of indigenous parasitic plant (*Viscum verrocosum*) in reducing faecal egg counts in female Tswana goats. Livest. Res. Rural Dev. 15, 1-6.

Makkar, H.P.S., Blummel, M., Borowy, N.K., Becker, K., 1993. Gravimetric determination of tannins and their correlations with chemical and protein precipitation method. J. Sci. Food. Agric. 61, 161-165.

Manolaraki, F., Sotiraki, S., Stefanakis, A., Skampardonis, V., Volanis, M., Hoste, H., 2010. Anthelmintic activity of some Mediterranean browse plants against parasitic nematodes. Parasitology 137, 685-696.

Marie-Magdeleine, C., Mahieu, M., Philibert, L., Despois, P., Archimede, H., 2010. Effect of cassava (*Manihot esculenta*) foliage on nutrition, parasite infection and growth of lambs. Small Rumin. Res. 93, 10-18.

Mcneilly, T.N., Devaney, E., Matthews, J.B., 2009. *Teladorsagia circumcincta* in the sheep abomasum: defining the role of dendritic cells in T cell regulation and protective immunity. Parasite Immunol. 31, 347-356.

Min, B.R., Barry, T.N., Attwood, G.T., McNabb, W.C., 2003. The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: a review. Anim. Feed Sci. Technol. 106, 3-19.

Min, B.R., Pomroy, W.E., Hart, S.P., Saúl, T., 2004. The effect of short-term consumption of a forage containing condensed tannins on gastro-intestinal nematode parasite infections in grazing wether goats. Small Rumin. Res. 51, 279-283.

Min, B.R., Hart, S.P., Millar, D., Tomita, G.M., Loetz, E., Sahlu, T., 2005. The effect of grazing forage containing condensed tannins on gastro-intestinal parasite infection and milk composition in Angora does. Vet. Parasitol. 130, 105-113.

[MAFF] Ministry of Agriculture, Fisheries, and Food, 1978. Manual of veterinary parasitological techniques. London, United Kingdom: MAFF, Technical Bulletin No. 18. 129 p.

Moore, D.A., Terrill, T.H., Kouakou, B., Shaik, S.A., Mosjidis, J.A., Miller, J.E., Vanguru, M., Kannan, G., Burke, J.M., 2008. The effects of feeding sericea lespedeza hay on growth rate of goats naturally infected with gastrointestinal nematodes. J. Anim. Sci. 86, 2328-2337.

Moreno-Gonzalo, J., Ferre, I., Celaya, R., Frutos, P., Ferreira, L.M.M., Hervás, G., García, U., Ortega-Mora, L.M., Osoro, K., 2012. Potential use of heather to control gastrointestinal nematodes in goats. Small Rumin. Res. 103, 60-68.

Mupeyo, B., Barry, T.N., Pomroy, W.E., Ramírez-Restrepo, C.A., Lopez-Villalobos, N., Pernthaner, A., 2011. Effects of feeding willow (*Salix* spp.) upon death of established parasites and parasite fecundity. Anim. Feed Sci. Technol. 164, 8-20.

Niezen, J.H., Waghorn, G.C., Charleston, W.A.G., 1998. Establishment and fecundity of *Ostertagia circumcincta* and *Trichostrongylus colubriformis* in lambs fed lotus

(*Lotus pedunculatus*) or perennial ryegrass (*Lolium perenne*). Vet. Parasitol. 78, 13-21.

Osoro, K., Benito-Peña, A., Frutos, P., García, U., Ortega-Mora, L.M., Celaya, R., Ferre, I., 2007a. The effect of heather supplementation on gastrointestinal nematode infections and performance in Cashmere and local Celtiberic goats on pasture. Small Rumin. Res. 67, 184-191.

Osoro, K., Mateos-Sanz, A., Frutos, P., García, U., Ortega-Mora, L.M., Ferreira, L.M.M., Celaya, R., Ferre, I., 2007b. Anthelmintic and nutritional effects of heather supplementation on Cashmere goats grazing perennial ryegrass-white clover pastures. J. Anim. Sci. 85, 861-870.

Osoro, K., Celaya, R., Moreno-Gonzalo, J., Ferreira, L.M.M., García, U., Frutos, P., Ortega-Mora, L.M., Ferre, I., 2009. Effects of stocking rate and heather supplementation on gastrointestinal nematode infections and host performance in naturally infected Cashmere goats. Rangeland Ecol. Manage. 62, 127-135.

Paolini, V., Bergeaud, J.P., Grisez, C., Prevot, F., Dorchies, Ph., Hoste, H., 2003a. Effects of condensed tannins on goats experimentally infected with *Haemonchus contortus*. Vet. Parasitol. 113, 253-261.

Paolini, V., Dorchies, Ph., Hoste, H., 2003b. Effects of sainfoin hay on gastrointestinal nematode infections in goats. Vet. Rec. 152, 600-601.

Paolini, V., Frayssines, A., De La Farge, F., Dorchies, Ph., Hoste, H., 2003c. Effects of condensed tannins on established populations and on incoming larvae of *Trichostrongylus colubriformis* and *Teladorsagia circumcincta* in goats. Vet. Res. 34, 331-339.

Paolini, V., De La Farge, F., Prevot, F., Dorchies, Ph., Hoste, H., 2005. Effects of the repeated distribution of sainfoin hay on the resistance and the resilience of goats naturally infected with gastrointestinal nematodes. Vet. Parasitol. 127, 277-283.

Pomroy, W.E., Adlington, B.A., 2006. Efficacy of short term feeding of sulla (*Hedysarum coronarium*) to young goats against a mixed burden of gastrointestinal nematodes. Vet. Parasitol. 136, 363-366.

Ríos-de Álvarez, L., Jackson, F., Greer, A.W., Grant, G., Jackson, E., Morrison, A.A., Huntley, J.F., 2012. Direct anthelmintic and immunostimulatory effects of oral dosing semi-purified phytohaemagglutinin lectin in sheep infected with *Teladorsagia circumcincta* and *Trichostrongylus colubriformis*. Vet. Parasitol. 187, 267-274.

SAS, 1999. SAS/STAT User´s Guide. V8. SAS Institute Inc., Cary, NC, USA.

Shaik, S.A., Terrill, T.H., Millar, J.E., Kouakou, B., Kannan, G., Kaplan, R.M., Burke, J.M., Mosjidis, J.A., 2006. Sericea lespedeza hay as a natural deworming agent against gastrointestinal nematode infections in goats. Vet. Parasitol. 139, 150-157.

Sokerya, S., Waller, P.J., Try, P., Hoglund, J., 2009. The effect of long-term feeding of fresh and ensiled cassava (*Manihot esculenta*) foliage on gastrointestinal nematode infections in goats. Trop. Anim. Health Prod. 41, 251-258.

Stear, M.J., Strain, S., Bishop, S.C., 1999. Mechanisms underlying resistance to nematode infection. Int. J. Parasitol. 29, 51–56.

Terrill, T.H., Mosjidis, J.A., Moore, D.A., Shaik, S.A., Millar, J.E., Burke, J.M., Muir, J.P., Wolfe, R., 2007. Effect of pelleting on efficacy of sericea lespedeza hay as a natural dewormer in goats. Vet. Parasitol. 146, 117-122.

Terrill, T.H., Dykes, G.S., Shaik, S.A., Miller, J.E., Kouakou, B., Kannan, G., Burke, J.M., Mosjidis, J.A., 2009. Efficacy of sericea lespedeza hay as a natural dewormer in goats: Dose titration study. Vet. Parasitol. 163, 52-56.

Tzamaloukas, O., Athanasiadou, S., Kyriazakis, I., Jackson, F., Coop, R.L., 2005. The consequences of short-term grazing of bioactive forages on established adult and incoming larvae populations of *Teladorsagia circumcincta* in lambs. Int. J. Parasitol. 35, 329-335.

Valcárcel, F., Romero, C.G., 1999. Prevalence and seasonal pattern of caprine trichostrongyles in a dry area of central Spain. J. Vet. Med. Ser. B-Infect. Dis. Vet. Public Health 46, 673-681.

Waller, P.J., 2006. Sustainable nematode parasite control strategies for ruminant livestock by grazing management and biological control. Anim. Feed Sci. Technol. 126, 277–289.

Waller, P.J., Thamsborg, S.M, 2004. Nematode control in 'green' ruminant production systems. Trends Parasitol. 20, 493-497.

CAPÍTULO IV

Experimentos *in vitro*

***In vitro* effect of heather extracts on *Trichostrongylus colubriformis* eggs, larvae and adults**

J. Moreno-Gonzalo [a], F. Manolaraki [b,c], P. Frutos [d], G. Hervás [d], R. Celaya [e], K. Osoro [e], L.M. Ortega-Mora [a], H. Hoste [b,c], I. Ferre [a,*]

[a] *SALUVET Group, Department of Animal Health, Faculty of Veterinary, Complutense University of Madrid, Ciudad Universitaria s/n, 28040-Madrid, Spain*

[b] *UMR 1225 INRA/ENVT, Ecole Nationale Vétérinaire de Toulouse – 23 Chemin des Capelles, 31076 Toulouse Cedex, France*

[c] *Université de Toulouse, ENVT, UMR 1225, F-31076 Toulouse, France*

[d] *Instituto de Ganadería de Montaña, CSIC-ULE, Finca Marzanas, 24346-León, Spain*

[e] *Servicio Regional de Investigación y Desarrollo Agroalimentario (SERIDA), Área de Sistemas de Producción Animal, 33300-Villaviciosa, Asturias, Spain*

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Abstract

This study was carried out to evaluate the *in vitro* effects of different heather species on *Trichostrongylus colubriformis* eggs, larvae and adult worms, and obtain scientific evidence to attribute these effects to the action of their phenolic compounds and/or tannins. Total phenolic extracts of three heather species (*Calluna vulgaris*, *Erica cinerea*, and *Erica umbellata*) and an equal mixture of these three extracts were tested *in vitro* in the three development stages of *T. colubriformis* using an egg hatching assay (EHA), larval exsheathment inhibition assay (LEIA), and adult motility inhibition assay (AMIA). The egg hatching rate was measured after incubation with heather extracts for 48 h at 25°C. Infective third-stage larvae (L3) were incubated for 3 h at 25°C with heather extracts. The evolution of artificial exsheathment over time was measured with repeated observations at 20-min intervals for 60 min. Adult worms were obtained from one donor goat and incubated with the extracts at 37°C for 5 days in 48-multiwell plates. Worm motility was measured at 0, 19, 24, 43, 48, 67, 72, 96 and 115 h after the beginning of the experiment. The extracts were tested at concentrations of 75, 150, 300, 600 and 1200 µg/ml. All extracts significantly ($P<0.001$) inhibited egg hatching and the effect was dose dependent. All extracts inhibited or delayed the exsheathment of *T. colubriformis* L3, and the effect was dose dependent for *C. vulgaris*. Incubation with heather extracts induced a reduction in adult worm motility compared to control, although significant ($P<0.05$) differences were only found at the highest concentrations. Additional studies showed that purified tannins of the same heather species disturbed *T. colubriformis* larval exsheathment. All these results confirm the anthelmintic properties of heather against *T. colubriformis*, and suggest that not only tannins but also some other phenolic compounds might be involved.

Keywords: Trichostrongylus colubriformis; Heather; *Erica* spp.; *Calluna vulgaris*; Egg hatching assay; Larval exsheathment inhibition assay; Adult motility inhibition assay.

1. Introduction

Gastrointestinal nematode infections are considered an important disease challenge in ruminants at pasture worldwide (Stear et al., 2007). Concerns regarding drug residue in livestock products and the development of resistant nematode populations to the most frequently used anthelmintic drugs have stimulated the search for alternative control strategies (Waller, 2006). The use of bioactive plants, mainly those including tannins, has received special attention (Hoste et al., 2006). The anthelmintic effect of these plants is suspected to be related to the ability of tannins to form complexes with parasite proteins. Tannins seem to affect different biological processes of nematodes depending on where and how the tannins bind to various nematode structures (Brunet et al., 2011).

Recently, numerous *in vitro* studies have been carried out to screen the anthelmintic effect of a range of plants or forages, especially in different *Haemonchus contortus* development stages (Manolaraki et al., 2010; Katiki et al., 2011; Kamaraj et al., 2011; Oliveira et al., 2009, 2011; Cala et al., 2012; Hussain et al., 2011). However, *in vitro* studies with other nematode species, such as *Trichostrongylus colubriformis* are less numerous (Molan et al., 2000b, 2002, 2003, 2004; Paolini et al., 2004; Brunet et al., 2007). *In vitro* bioassays have the advantage of providing a rapid, simple and low-cost means to test plants or other products for their anthelmintic potential. However, variations in results have been found according to the parasitic stage, particularly depending on whether adult worms, infective larvae, or eggs are submitted to a tannin-rich environment (Paolini et al., 2003).

Previous *in vivo* experiments performed outdoor and indoor showed the anthelmintic activity of heather supplementation in goats infected with *T. circumcincta* and *T. colubriformis* (Moreno-Gonzalo et al., 2012, 2013). Therefore, the purpose of this study was to evaluate the anthelmintic activity of three heather species (*Calluna vulgaris*, *Erica cinerea*, and *Erica umbellata*), which are usually eaten by goats in northern Spain, on the eggs, larvae and adult worms of *T. colubriformis* using different *in vitro* assays, with the final goal of confirming that the anthelmintic effects are due to their phenolic and/or tannin content. Only Bahuaud et al. (2006) tested the anthelmintic effect of heather on *T. colubriformis* larval exsheathment after incubation with *Erica erigena* extracts, but they found no significant delay in the process of exsheathment. To our knowledge this is the first time that the anthelmintic activity of the extracts of a plant was tested in the three development stages of *T. colubriformis*. It has been only tested in *H. contortus* (Hounzangbe-Adote et al., 2005; Marie-Magdeleine et al., 2009 and 2010; Bachaya et al., 2009). And it is the first time that the anthelmintic effect of a plant observed in field experiments has been confirmed in controlled trials and finally its extracts tested on the three stages of the most important gastrointestinal nematode species of goats in temperate areas (Moreno-Gonzalo et al., 2012, 2013).

2. Materials and Methods

2.1. Experimental design

The effects of four heather (*C. vulgaris*, *E. cinerea*, *E. umbellata* and a equal mixture of all three) total phenolic extracts on the three main stages of the *T. colubriformis* cycle, i.e., eggs, third-stage larvae (L3) and adult worms, were measured using three *in vitro* assays: the egg hatching assay (EHA), larval exsheathment inhibition assay (LEIA), and adult motility inhibition assay (AMIA), respectively. Moreover, LEIA was performed using purified tannin extracts to confirm the role of tannins in the anthelmintic effect of heather.

2.2. Heather extracts

Non-purified extracts of total phenolics were obtained according to the procedures described by Makkar et al. (1993). First, pigments and fats were removed from the dried plant material using diethyl ether containing 1% acetic acid. Total phenols were then extracted with 70% aqueous acetone. This extract contains mainly tannins but also other phenolic compounds. After freeze-drying these crude extracts, tannin purifications was performed using a slurry of Sephadex LH-20 in 80% ethanol, with sequential washes with 95% ethanol and 50% aqueous acetone, to selectively separate tannins from other compounds, plus extraction with ethyl acetate (Asquith and Butler, 1985; Hagerman, 1991). The resulting extract contained only purified tannins.

2.3. Parasites

Eggs, L3 larvae and adults of *T. colubriformis* for EHA, LEIA and AMIA were obtained from a donor goat experimentally infected *per os* with a pure strain of 6,000 *T. colubriformis* L3. For LEIA, batches of 2- to 3-month old L3 larvae were used. For AMIA, four weeks after infection, the goat was euthanized, and immediately after death, the small intestine was collected, opened, briefly washed and placed in a Baermann apparatus with saline at 37°C. After 2 h, worms that had migrated into the saline were collected. The *T. colubriformis* strain was susceptible to the main anthelmintic drugs.

2.4. Egg hatching assay

This method was based on a modification of the EHA performed to measure anthelmintic efficacy (WAAVP recommendations, Coles et al., 1992). *T. colubriformis* eggs were extracted from faeces by repeated centrifugation and washing and distributed in 24-multiwell plates at a density of 100 eggs per well in phosphate buffered saline (PBS; 0.1 M phosphate, 0.05 M NaCl, pH 7.2). Increasing concentrations of plant extracts (75, 150, 300, 600, and 1200 µg dry matter/ml) were obtained from lyophilised extracts dissolved in PBS and added to each well. Each concentration was tested on four replicates. Eggs were incubated for 48 h at 24°C. Subsequently, the number of larvae per well was counted, and the percentage of eggs hatched was determined as the ratio between the number of larvae and the number of eggs deposited per well. A mean percentage of hatching was calculated for each concentration of the different plant extracts.

2.5. Larval artificial exsheathment assay

First, batches of 1500 ensheathed L3 were incubated for 3 h with each of the four heather extracts at concentrations of 75, 150, 300, 600 and 1200 µg/ml for the raw extracts and of only 15, 30, 60 and 120 µg/ml (10-fold less concentrated) for the purified tannins diluted in PBS. PBS was used to avoid interference from any non-specific effect due to pH change. After incubation, the larvae were washed and centrifuged three times in PBS. Then, the larvae were submitted to the artificial process of exsheathment by contact with a solution of sodium hypochlorite (2% w/v) and sodium chloride (16.5% w/v) diluted 1 to 300 in PBS. The kinetics of larval exsheathment according to the experimental treatments was then measured by microscopic observation, and exsheathed larvae were identified at a magnification of x200 by regular examination at 0, 20, 40 and 60 min after contact with the hypochlorite solution. Four replicates were used for each plant per time and concentration to examine the changes in the proportion of exsheathed larvae over time.

2.6. Adult motility inhibition assay

Adult *T. colubriformis* were placed in a 48-multiwell plate at a concentration of two worms per well. The plates were maintained at 37°C throughout. The worms were first washed for 1 h in PBS with 4% penicillin and streptomycin. Subsequently, 1 ml of the different concentrations (from 75 to 1200 µg/ml) of heather extracts diluted in PBS (with 4% penicillin and streptomycin) was added to the wells. Levamisole (concentrations of 0.125%, 0.25%, 0.5% and 1%) was used as an anthelmintic control, and PBS negative controls were included on each plate (eight worms per treatment). For each treatment, measurements were made on four replicates per plate. The supernatant was changed every 24 h. The motility of adult worms was noted by careful observation under a stereomicroscope at x40 magnification after 0, 19, 24, 43, 48, 67, 72, 96 and 115 h. Death or paralysis of worms was ascertained by absence of motility for an observation period of 10 s. At each time, the motility index was calculated as the ratio between the number of mobile (live) worms and the total number of worms. The assay was considered completed when more than 90% of worms had died in the control (PBS).

2.7. Statistical analyses

In EHA, the significance of differences in the means of the proportion of unhatched eggs rates between treatments (dose) was assessed for each plant extract by the general linear model (GLM) using Systat 9 software (SPSS Ltd.). In LEIA, for both the total phenolic heather extracts and the purified tannins, the statistical differences in mean of the exsheathment rates, based on the results of the four replicates, depending on treatment (plant extract), dose and time, were performed for each by the GLM procedure using the Systat 9 software (SPSS Ltd.). The extract concentration required to inhibit 50% of egg hatching or 50% of L3 exsheathment (EC_{50}) was calculated using the POLO plus statistical program. In AMIA, for each treatment (plant extract and dose) the number of immobile worms was recorded over time. Survival was analysed with the non parametric stratified Cox regression test using Systat 9 software. For the three *in vitro* assays, the dose-response relationship, according to a regression, was determined considering the statistical level of significance as $P<0.05$.

3. Results

3.1. Egg hatching assay

The mean hatching rate measured in the negative control was 75.1%. A dose dependent effect ($P<0.001$) was observed on the egg hatching rate when the eggs were incubated with the total phenolic extracts of the three species of heather and the mixture of all extracts (Figure 1). *E. umbellata* extract appeared to be the most effective based on EC_{50} (Table 1).

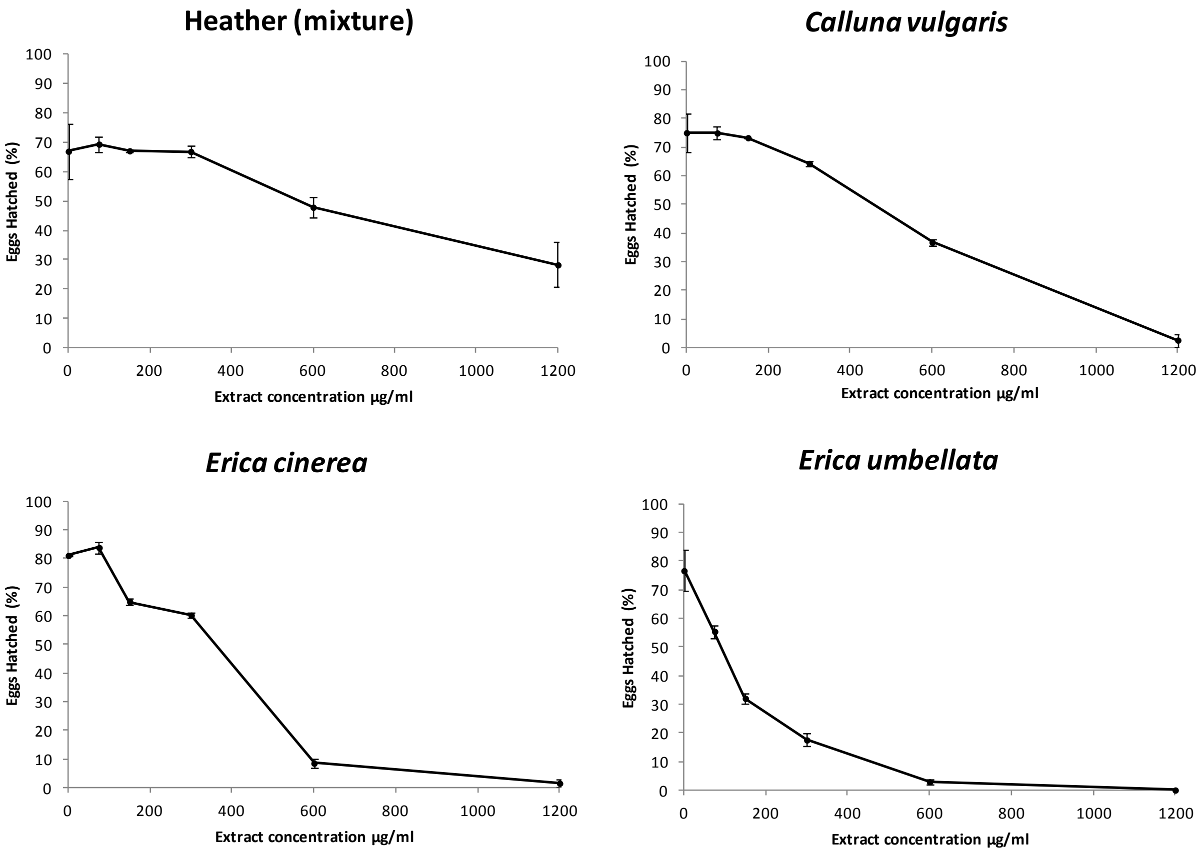

Figure 1. Effect of different concentrations of heather extracts (mixture of heather extracts, *Calluna vulgaris*, *Erica cinerea* and *Erica umbellata*) on the proportion of *T. colubriformis* eggs hatched *in vitro*. Each point represents the mean (±SD) of four replicates per extract and concentration**.**

Table 1. Extract concentration required to inhibit 50% of egg hatching (EC_{50}), and lower and upper 90% limits for the raw heather (*Calluna vulgaris*, *Erica cinerea*, *Erica umbellata* and a mixture of all three) extracts for EHA.

	EC_{50} (µg/mL)	Lower limit 90% (µg/mL)	Upper limit 90% (µg/mL)
Calluna vulgaris	521.6	454.4	581.7
Erica cinerea	335.7	277.3	390.7
Erica umbellata	120.9	111.8	130.1
Mixture	791.3	659.9	994.9

3.2. Larval artificial exsheathment assay

In the PBS control, more than 90% of larvae were exsheathed at 60 minutes after deposition of sodium chloride solution. Significant (P<0.001) differences were found in the exsheathment process between the larvae incubated with PBS and those incubated with *E. cinerea*, *E. umbellata* and the mixture of the three heather raw extracts (Figure 2a), but they were not dose dependent. Larvae exsheathment was delayed and almost totally inhibited when larvae were incubated with the highest concentration of extract. The EC_{50} values suggest that raw extracts of *C. vulgaris* were much less effective than the two *Erica* species and the mixture (Table 2). Regarding the purified tannins and despite the much lower doses tested, *E. umbellata* and *E. cinerea* extracts significantly (P<0.001) or almost significantly (P<0.08) reduced L3 exsheathment, respectively. In both cases, the effect was dose dependent (P<0.05). *C. vulgaris* and the mixture of heather extracts showed a significant (P<0.05) dose effect (Figure 2b). Again, EC_{50} values suggested that the mixture of tannins was more active than purified tannins of *C. vulgaris* and *E. cinerea* alone (Table 2).

3.3. Adult motility inhibition assay

A significant (P<0.05) reduction of worm motility was observed when adult nematodes were incubated with the highest concentration (1200 µg/ml) of each extract and the mixed heather extract at 600 and 150 µg/ml (Figure 3).

4. Discussion

The increasing use of plants, forages or plant extracts as phytomedicines or nutraceuticals is becoming popular as an alternative or complement to synthetic drugs (Sandoval-Castro et al., 2012). In the present study, the objective was to evaluate the *in vitro* anthelmintic effects of phenolic extracts of different heather species, *C. vulgaris*, *E. cinerea* and *E. umbellata*, and a combination of all three on *T. colubriformis* eggs, larvae and adults to be able to attribute previous reports obtained under grazing and/or controlled *in vivo* conditions (Moreno et al., 2012, 2013) to the action of their phenolic compounds and/or tannins.

Table 2. Extract concentration required to inhibit 50% of infective larvae exsheathment (EC_{50}), and lower and upper 90% limits for the different non-purified and purified heather (*Calluna vulgaris*, *Erica cinerea*, *Erica umbellata* and a mixture of all three) extracts for LEIA.

	EC_{50} (µg/mL)	Lower limit 90% (µg/mL)	Upper limit 90% (µg/mL)
Raw extracts			
Calluna vulgaris	574.8	459.8	723.3
Erica cinerea	<150		
Erica umbellata	<150		
Mixture	<150		
Purified extracts			
Calluna vulgaris	70.14	59.6	80.2
Erica cinerea	108.2	84.5	167.2
Erica umbellata	NC		
Mixture	39.03	27.4	63.4

NC: not calculated

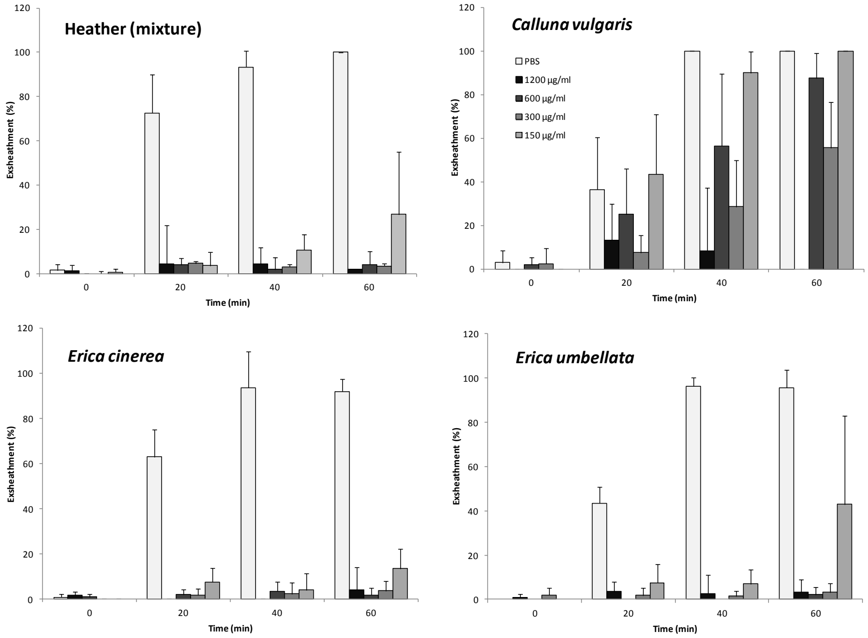

Figure 2a

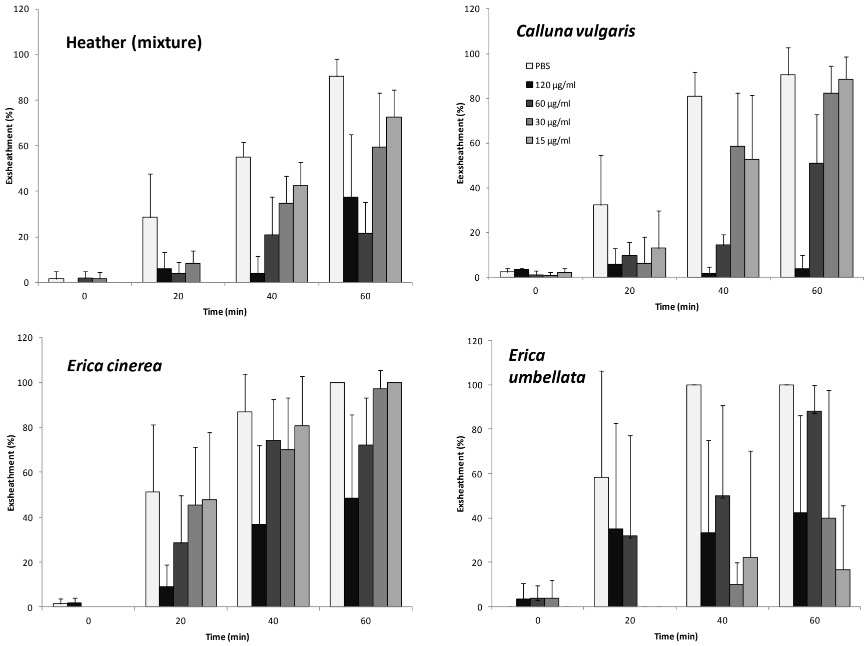

Figure 2b

Figure 2. Effect of different concentrations of heather extracts (mixture of heather extracts, *Calluna vulgaris*, *Erica cinerea* and *Erica umbellata*) on *T. colubriformis* third-stage larvae after 3 h of incubation and artificial *in vitro* exsheathment provoked by the addition of a 2% solution of hypochloride. Figures 2a and 2b show the results of incubation with total phenolic compounds and purified tannin heather extracts, respectively. Each point represents the mean (±SD) of four replicates per extract and concentration**.**

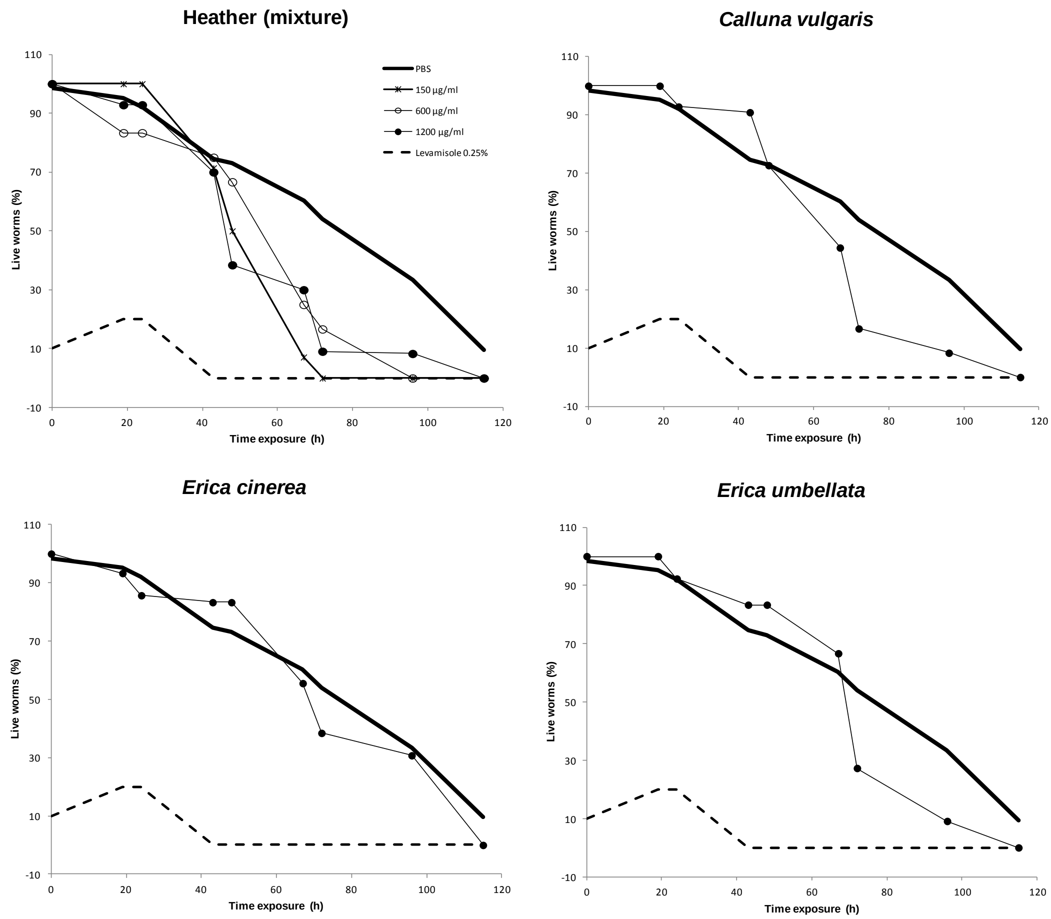

Figure 3. Time-anthelmintic activity of heather extracts compared with positive (levamisole 0.25%) and negative (PBS) controls on *T. colubriformis* adult worms, expressed as the percentage of live worms (mobile worms) of the total exposed worms. Only the concentrations showing significant (P<0.05) effect are included.

Infective trichostrongylid larvae are not able to infect the host without exsheathment (Hertzberg et al., 2002). Incubation with the total phenolic heather extracts (*C. vulgaris*, *E. cinerea*, *E. umbellata* and a mixture of all three) interfered with the *T. colubriformis* L3 exsheathment. The results showed that all extracts at 1200 µg/ml blocked the exsheathment process. Similar results were obtained by Alonso-Díaz et al. (2008) when *T. colubriformis* infective larvae were incubated with total phenolic extracts of four tropical plants (*Acacia pennatula*, *Lysiloma latisiliquum*, *Piscidia piscipula*, and *Leucaena leucocephala*). However, Bahuaud et al. (2006) did not find any significant effect on *T. colubriformis* larval exsheathment when the larvae were incubated with phenolic extracts of another heather species (*E. erigena*). The discrepancies in these results could be associated with not only the different compound extracts but also different methodologies and solvents used for extraction. The inhibition of *T. colubriformis* larvae exsheathment by heather extracts could explain the reduction in larval establishment reported in Cashmere goats experimentally infected with *T. colubriformis* and fed heather (Moreno-Gonzalo et al., 2012). The exsheathment rate reduction observed when larvae were incubated with the pure tannin extracts (even at much lower concentrations, as mentioned before) suggests that tannins were responsible, at least in part, for the inhibitory effect of heather extracts on *T. colubriformis* L3 exsheathment. However, according to these results, the anthelmintic effect of other non-tannin phenolic compounds cannot be ruled out. Ultrastructural changes in different regions through a direct interaction between bioactive plant compounds (i.e., tannins) and nematode structures have been reported (Brunet et al., 2011) and could explain the deleterious effects of heather on larval exsheathment.

All extracts at 1200 µg/ml (the highest concentration used) significantly reduced the survival (measured as the existence of worm motility) of *T. colubriformis* adult worms. Paolini et al. (2004) reported similar results with *Onobrychis viciifolia* (sainfoin), *Rubus fructicosus* (brambles), *Quercus robur* (oak), and *Corylus avellana* (hazel tree) extracts on *T. colubriformis*, *T. circumcincta* and *H. contortus* adult worms. The increase in adult *T. colubriformis* mortality when they are incubated with heather extracts could explain the reduction in faecal gastrointestinal nematode egg counts and worm burden previously shown in Cashmere goats experimentally infected with *T. colubriformis* and fed heather (Moreno-Gonzalo et al., 2012). Nevertheless, this reduction in fecal egg counts and worm burden observed in field experiment as well, could also be explained by the effect of heather on the exsheathment of incoming larvae, which seems to be the most affected stage by the action of heather.

In vitro assays seem to be useful tools to screen bioactive plants for anthelmintic activity (Kotze et al., 2009; Manolaraki et al., 2010; Jackson and Hoste, 2010). However, some authors have found some discrepancies in the results depending on the parasitic stage or even at the same stage depending on the assay used. Alonso-Díaz et al. (2008) found important differences between the larval migration inhibition assay and LEIA using the same extracts and nematode strains. Molan et al. (2002) observed that tannins were more potent inhibitors of larval development and egg hatching than larval motility. In any case, the effects of some heather extracts on *T. colubriformis* eggs, infective larvae and adult worms observed under *in vitro* conditions confirm the anthelmintic activity. Interestingly, the mixture of extracts appeared to be a more potent inhibitor of larval exsheathment than single extracts, therefore heather consumption could contribute to the control of gastrointestinal nematodes by reducing the contamination of pastures and infection rate of small ruminants (Moreno-Gonzalo et al., 2012).

It is important to emphasise that some cautions must be taken when transposing *in vitro* results to *in vivo* conditions. The chemical components of the plants could be modified after passage in the gastrointestinal tract. In particular, the ruminal conditions could affect tannins because of bacteria degradation (Makkar, 2003) (although the condensed tannins, which are more abundant in heather, are not degraded in the rumen) and/or the formation of complexes with proteins. The heather extract concentrations used in the present experiments tried to simulate those reported as within the physiological range of total condensed tannin concentrations found in the abomasal digesta in sheep fed diets containing tannins (Terrill et al., 1994; Molan et al., 2000a). However, it must be stressed that the lack of standardisation of the analysis of this large group of phenolic compounds and the use of different standards to express tannin concentration (i.e., tannic acid, quebracho, internal standards extracted from the plants themselves) means that between-experiment comparisons can seldom be made (Álvarez del Pino et al., 2005; Frutos et al., 2008). Furthermore, although some tannin may be potentially toxic when consumed at high doses, heather intake did not present anti-nutritional effects on grazing goats (Frutos et al., 2008). The explanation of the fact that the most effective extract was the mixed heather extract (or at least, the extract with the most homogeneous results in the tree assays) could be that the composition of the extracts is different and the extract of mixed heather contains all the active compounds present in each one of the other three species extracts, but maybe at lower concentration. It is known that different condensed tannins monomers have different activities and the degree of polymerization or the number of free hydroxy groups is a key factor in the interactions with parasites (Brunet and Hoste, 2006). On the other hand, a synergic or antagonistic action of components in mixed heather extract is also possible (Copani et al., 2012).

In conclusion, the *in vitro* assays with heather phenolic extracts showed anthelmintic activity against eggs, larvae and adults of *T. colubrifomis* within a range of concentrations that could be reached in the digestive tract of ruminants. These results confirm those obtained in *in vivo* trials and, although the purified extracts were only tested with LEIA, it allows to attribute the anthelmintic effects to the action of tannins and/or other non-tannin phenolic compounds. In addition, they would confirm that heather could be used to help to control gastrointestinal nematodes in grazing ruminants.

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Conflict of interest

None.

References

Alonso-Díaz, M.A., Torres-Acosta, J.F., Sandoval-Castro, C.A., Capetillo-Leal, C., Brunet, S., Hoste, H., 2008. Effects of four tropical tanniniferous plant extracts on the inhibition of larval migration and the exsheathment process of *Trichostrongylus colubriformis* infective stage. Vet. Parasitol. 153, 187-192.

Álvarez del Pino, M.C., Hervás, G., Mantecón, A.R., Giráldez, F.J., Frutos, P., 2005. Comparison of biological and chemical methods, and internal and external standards, for assaying tannins in Spanish shrub species. J. Sci. Food Agric. 85, 583-590.

Asquith, T.N., Butler L.G., 1985. Use of dye-labeled protein as spectrophotometric assay for protein precipitants such as tannin. J. Chem. Ecol. 11, 1535-1544.

Bachaya, H. A., Iqbal, Z., Khan, M. N., Sindhu, Z.U., Jabbar, A., 2009. Anthelmintic activity of *Ziziphus nummularia* (bark) and *Acacia nilotica* (fruit) against Trichostrongylid nematodes of sheep. J. Ethnopharmacol. 123, 325-329.

Bahuaud, D., De Montellano, C.M.O., Chauveau , S., Prevot, F., Torres-Acosta, F., Fouraste, I., Hoste, H., 2006. Effects of four tanniferous plant extracts on the *in vitro* exsheathment of third-stage larvae of parasitic nematodes. Parasitology 132, 545-554.

Brunet, S., Hoste, H., 2006. Monomers of condensed tannins affect the larval exsheathment of parasitic nematodes of ruminants. J. Agric. Food Chem. 54, 7481-7487.

Brunet, S., Aufrere, J., El Babili, F., Fouraste, I., Hoste, H., 2007.The kinetics of exsheathment of infective nematode larvae is disturbed in the presence of a tannin-rich plant extract (sainfoin) both *in vitro* and *in vivo*. Parasitology 134, 1253-1262.

Brunet, S., Fourquaux I., Hoste H., 2011. Ultrastructural changes in the third-stage, infective larvae of ruminant nematodes treated with sainfoin (*Onobrychis viciifolia*) extract. Parasitol. Int. 60, 419–424.

Cala, A.C., Chagas, A.C.S., Oliveira, M.C.S., Matos, A.P., Borges, L.M.F., Sousa, L.A.D., Souza, F.A., Oliveira, G.P, 2012. *In vitro* anthelmintic effect of *Melia azedarach* L. and *Triclinia claussenii* C. against sheep gastrointestinal nematodes. Exp. Parasitol. 130, 98-102.

Coles, G.C., Bauer, C., Borgsteede, F., Geerts, S., Klei, T.R., Taylor, M.A., Waller, P.J., 1992. World Association for Advancement in Veterinary Parasitology (WAAVP) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. Vet. Parasitol. 44, 35-43.

Copani, G., Hall, J.O., Miller, J., Priolo, A., Villalba, J.J., 2012. Plant secondary compounds as complementary resources: are they always complementary?. Oecologia, doi 10.1007/s0042-012-2551-1.

Frutos, P., Moreno-Gonzalo, J., Hervás, G., García, U., Ferreira, L.M.M., Celaya, R., Toral, P.G., Ortega-Mora, L.M., Ferre, I., Osoro, K., 2008. Is the anthelmintic effect of heather supplementation to grazing goats always accompanied by anti-nutritional effects? Animal 2, 1449-1456.

Hagerman, A.E., 1991. Tannin Analysis. Miami University, Oxford, OH, USA.

Hertzberg, H., Huwyler, U., Kohler, L., Rehbein, S., Wanner, M., 2002. Kinetics of exsheathment of infective ovine and bovine strongylid larvae *in vivo* and *in vitro*. Parasitology 125, 65-70.

Hoste, H., Jackson, F., Athanasiadou, S., Thamsborg, S.M., Hoskin, S.O., 2006. The effects of tannin-rich plants on parasitic nematodes in ruminants. Trends Parasitol. 22, 253–261.

Hounzangbe-Adote, M.S., Paolini, V., Fouraste, I., Moutairou, K., Hoste, H., 2005. In vitro effects of four tropical plants on three life-cycle stages of the parasitic nematode, *Haemonchus contortus*. Res. Vet. Sci. 78, 155-160.

Hussain, A., Khan, M.N., Iqbal, Z., Sajid, M.S., Khan, M.K., 2011. Anthelmintic activity of *Trianthema portulacastrum* L. and *Musa paradisiaca* L. against gastrointestinal nematodes of sheep. Vet. Parasitol. 179, 92-99.

Jackson, F., Hoste, H., 2010. *In vitro* methods for the primary screening of plant products for direct activity against ruminant gastrointestinal nematodes. In: Vercoe, P.E., Makkar, H.P.S., Schlink, A.C. (Eds.), *In vitro* screening plant resources for extra nutritional attributes in ruminants: nuclear and related methodologies. FAO/IAEA Springer Edition, pp. 24-45.

Kamaraj, C., Rahuman A.A., 2011. Efficacy of anthelmintic properties of medicinal plant extracts against *Haemonchus contortus*. Res. Vet. Sci. 91, 400-404.

Katiki, L.M., Chagas, A.C.S., Bizzo, H.R., Ferreira, J.F.S., Amarante, A.F.T., 2011. Anthelmintic activity of *Cymbopogon martinii*, *Cymbopogon schoenanthus* and *Mentha piperita* essential oils evaluated in four different *in vitro* tests. Vet. Parasitol. 183, 103-108.

Kotze, A.C., O'Grady, J., Emms, J., Toovey, A.F., Hughes, S., Jessop, P., Bennell, M., Vercoe, P.E., Revell, D.K., 2009. Exploring the anthelmintic properties of Australian native shrubs with respect to their potential role in livestock grazing systems. Parasitology 136, 1065-1080.

Makkar, H.P.S., Blümmel, M., Borowy, N.K. and Becker, K., 1993. Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. J. Sci. Food Agric. 61, 161-165.

Makkar, H.P.S. 2003. Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. Small Rumin. Res. 49, 241-256.

Manolaraki, F., Sotiraki, S., Stefanakis, A., Skampardonis, V., Volanis, M., Hoste, H., 2010. Anthelmintic activity of some Mediterranean browse plants against parasitic nematodes. Parasitology 137, 685-696.

Marie-Magdeleine, C., Hoste, H., Mahieu, M., Varo, H., Archimede, H., 2009. *In vitro* effects of *Cucurbita moschata* seed extracts on *Haemonchus contortus*. Vet. Parasitol. 161, 99-105.

Marie-Magdeleine, C., Udino, L., Philibert, L., Bocage, B., Archimede, H., 2010. *In vitro* effects of Cassava (*Manihot esculenta*) leaf extracts on four development stages of *Haemonchus contortus*. Vet. Parasitol. 173, 85-92.

Molan, A.L., Alexander, R.A., Brookes, I.M., Mc Nabb, W.C., 2000a. Effects of an extract from sulla (*Hedysarum coronarium*) containing condensed tannins on the migration of three sheep gastrointestinal nematodes *in vitro*. Proc. N. Z. Soc. Anim. Prod. 60, 21-25.

Molan, A.L., Waghorn, G.C., Min, B.R., McNabb, W.C., 2000b. The effect of condensed tannins from seven herbages on *Trichostrongylus colubriformis* larval migration *in vitro*. Folia Parasitol. (Praha), 47, 39-44.

Molan, A.L., Waghorn, G.C., McNabb, W.C., 2002. Effect of condensed tannins on egg hatching and larval development of *Trichostrongylus colubriformis in vitro*. Vet. Rec. 150, 65-69.

Molan, A.L., Meagher, L.P., Spencer, P.A., Sivakumaran, S., 2003. Effect of flavan-3-ols on *in vitro* egg hatching, larval development and viability of infective larvae of *Trichostrongylus colubriformis*. Int. J. Parasitol. 33, 1691-1698.

Molan, A.L. Sivakumaran, S., Spencer, P.A., Meagher, L.P., 2004. Green tea flavan-3-ols and oligomeric proanthocyanidins inhibit the motility of infective larvae of *Teladorsagia circumcincta* and *Trichostrongylus colubriformis in vitro*. Res. Vet. Sci. 77, 239-243.

Moreno-Gonzalo, J., Ferre, I., Celaya, R., Frutos, P., Ferreira, L.M.M., Hervás, G., García, U., Ortega-Mora L.M., Osoro, K., 2012. Potential use of heather to control gastrointestinal nematodes in goats. Small Rumin. Res. 103, 60–68.

Moreno-Gonzalo, J., Osoro, K., García, U., Frutos, P., Celaya, R., Ferreira, L.M.M., Ortega-Mora L.M., Ferre, I., 2013. Effect of the consumption of heather on incoming larvae and established population of *Teladorsagia circumcincta* in experimentally infected Cashmere goats. Vet. Parasitol., doi 10.1016/j.vetpar.2013.02.005.

Oliveira, L.M.B., Bevilaqua, C.M.L., Costa, C.T.C., Macedo, I.T.F., Barros, R.S., Rodrigues, A.C.M., Camurça-Vasconcelos, A.L.F., Morais, S.M., Lima, Y.C., Vieira, L.S., Navarro, A.M.C., 2009. Anthelmintic activity of *Cocos nucifiera* L. against sheep gastrointestinal nematodes. Vet. Parasitol. 159, 55-59.

Oliveira, L.M.B., Bevilaqua, C.M.L., Macedo, I.T.F., Morais, S.M., Machado, L.K.A. Campello, C.C., Mesquita, M.D., 2011. Effects of *Myracrodruon urundeuva* extracts on egg hatching and larval exsheathment of *Haemonchus contortus*. Parasitol. Res. 109, 893-898.

Paolini, V., Frayssines, A., De La Farge, F., Dorchies, P., Hoste, H., 2003. Effects of condensed tannins on established populations and on incoming larvae of *Trichostrongylus colubriformis* and *Teladorsagia circumcincta* in goats. Vet. Res. 34, 331-339.

Paolini, V., Fouraste, I., Hoste, H., 2004. *In vitro* effects of three woody plant and sainfoin extracts on 3rd-stage larvae and adult worms of three gastrointestinal nematodes. Parasitology 129, 69-77.

Sandoval-Castro, C.A., Torres-Acosta, J.F.J., Hoste, H., Salem, A.Z.M., Chan-Pérez, J.I., 2012. Using plant bioactive materials to control gastrointestinal tract helminths in livestock. Anim. Feed Sci. Technol. 176, 192-201.

Stear, M. J., Doliglska, M., Donskow-Schmelter, K., 2007. Alternatives to anthelmintics for the control of nematodes in livestock. Parasitology 134, 139-151.

Terrill, T.H., Waghorn, G.C., Woolley, D.J., McNabb, W.C., Barry, T.N., 1994. Assay and digestion of C-14-labeled condensed tannins in the gastrointestinal-tract of sheep. Br. J. Nutr. 72, 467-477.

Waller, P.J., 2006. Sustainable nematode parasite control strategies for ruminant livestock by grazing management and biological control. Anim. Feed Sci. Technol. 126, 277–289.

In vitro effect of heather (Ericaceae) extracts on different development stages of *Teladorsagia circumcincta* and *Haemonchus contortus*

J. Moreno-Gonzalo [a], F. Manolaraki [b,c], P. Frutos [d], G. Hervás [d], R. Celaya [e], K. Osoro [e], L.M. Ortega-Mora [a], H. Hoste [b,c], I. Ferre [a,*]

[a] *SALUVET Group, Department of Animal Health, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040-Madrid, Spain*

[b] *UMR 1225 INRA/ENVT, Ecole Nationale Vétérinaire de Toulouse – 23 Chemin des Capelles, 31076 Toulouse Cedex, France*

[c] *Université de Toulouse, ENVT, UMR 1225, F-31076 Toulouse, France*

[d] *Instituto de Ganadería de Montaña, CSIC-ULE, Finca Marzanas, 24346-León, Spain*

[e] *Servicio Regional de Investigación y Desarrollo Agroalimentario (SERIDA), Área de Sistemas de Producción Animal, 33300-Villaviciosa, Asturias, Spain*

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Abstract

The aim of the present study was to evaluate the *in vitro* effects of heather (Ericaceae) phenolic extracts on the abomasal nematodes *Teladorsagia circumcincta* and *Haemonchus contortus*. Extracts of three heather species (*Calluna vulgaris*, *Erica cinerea*, *Erica umbellata* and a balanced mixture of all three) were tested *in vitro* on different development stages of *T. circumcincta* (eggs, infective larvae and adult worms) and *H. contortus* (eggs and infective larvae) using an egg hatching assay (EHA), a larval exsheathment inhibition assay (LEIA) and an adult motility inhibition assay (AMIA). The egg hatching rate was measured after incubation with heather extracts for 48 h at 25°C. Ensheathed infective larvae were incubated for 3 h at 20°C with heather extracts. Artificial exsheathment was induced *in vitro* by adding hypochloride solution to the larval suspension. The progress of exsheathment over time was measured by repeated observations at 10-min (*T. circumcincta*) and 20-min (*H. contortus*) intervals for 60 min. Adult *T. circumcincta* worms were obtained from two donor goats and incubated with the extracts at 37°C for 3 days in 48-well multiwell plates. Worm motility was measured at 0, 19, 24, 43, 48, 67 and 72 h. The extracts were tested at concentrations of 75, 150, 300, 600 and 1,200 µg/mL. Incubation with *E. cinerea*, *E. umbellata* and mixed heather extracts had a significant ($P<0.01$) dose-dependent effect on *T. circumcincta* egg hatching. *H. contortus* egg hatching was significantly ($P<0.01$) inhibited only by the *E. cinerea* extract. All extracts had a significant ($P<0.01$) dose-dependent effect on the exsheathment of *T. circumcincta* and *H. contortus* infective larvae. The incubation with all heather extracts induced a reduction in adult *T. circumcincta* motility compared to the control, although significant ($P<0.05$) differences were only found at the highest concentration (1,200 µg/mL). The effect of the mixed extract was significant at all concentrations and significant effects were also observed for *C. vulgaris* and *E. umbellata* at 600 µg/mL. These results show anthelmintic properties of heather phenolic extracts against *T. circumcincta* and *H. contort*, thus confirming observations from previous *in vivo* studies.

Keywords: *Teladorsagia circumcincta*; *Haemonchus contortus*; Heather; *Erica* spp.; *Calluna vulgaris*; Egg hatching assay; Larval exsheathment inhibition assay; Adult motility inhibition assay; Phenolics

1. Introduction

Gastrointestinal nematode parasitism remains a major threat to efficient small ruminant production in pasture-based systems worldwide (Perry and Randolph, 1999). Infections by trichostrongyles are one of the main limitations for efficient sheep and goat production in temperate and tropical areas, especially when the abomasal parasite *Haemonchus contortus* is involved (Hoste et al., 2012). For several decades, the control of this parasitic condition has been achieved mainly through intensive chemoprophylaxis, based on the repeated use of synthetic anthelmintics. However, the development of anthelmintic resistant strains in many nematode species is a worldwide phenomenon, making the control of these parasites increasingly difficult (Kaplan, 2004). Additionally, the increasing consumer demand to reduce the use of chemical substances in the farming industry has prompted active research on alternative or complementary solutions to chemotherapy for the control of gastrointestinal parasitism (Hoste and Torres-Acosta, 2011). The use of nutraceuticals, especially plants rich in tannins, has been suggested as a sustainable alternative for the control of gastrointestinal nematodes (Hoste et al., 2006). The role of other polyphenolic compounds, such as flavonoid glycosides, in anthelmintic activity has also been suggested (Barrau et al., 2005; Ademola et al., 2005).

Browser plants such as shrubs, trees or bushes could provide significant nutritional resources to animals in many small ruminant production systems, especially in goats (Papachristou et al., 2005). The possible anthelmintic activity of these plants (often rich in tannins and other phenolic compounds) included in the vegetation of rangelands is now receiving special attention (Akkari et al., 2008; Landau et al., 2010; Moreno-Gonzalo et al., 2012). The consumption of heather (Ericaceae) by grazing goats naturally infected with gastrointestinal nematodes has shown to be associated with lower faecal egg counts and a greater resilience of animals to infection (Osoro et al., 2007, 2009). This fact was confirmed in controlled trials with goats experimentally infected with *Trichostrongylus colubriformis* (Frutos et al., 2011) and *Teladorsagia circumcincta* (Moreno-Gonzalo et al., 2013). A significant decrease in the establishment of *T. circumcincta* infective larvae in goats that were fed heather was reported as early as six days post-infection, suggesting that heather could have some effect on *T. circumcincta* larvae exsheathment (Moreno-Gonzalo et al., 2013). Recently, *in vitro* studies showed the anthelmintic effects of heather phenolics on the different development stages of *T. colubriformis* (Moreno-Gonzalo et al., unpublished data). Bahuaud et al. (2006) also found that *Erica erigena* extracts exhibited anthelmintic activity on *H. contortus* but not on *T. colubriformis* larvae.

The objective of the present work was to test the *in vitro* effects of phenolic extracts from the heather species *Calluna vulgaris*, *Erica cinerea*, *Erica umbellata* and a balanced mixture of all three species on different development stages of *T. circumcincta* (eggs, infective larvae and adult worms) and *H. contortus* (eggs and infective larvae) to compare any effects of the heather species, parasite species and/or parasitic stage involved.

2. Materials and methods

2.1. Experimental design

The effects of phenolic extracts from three heather species (i.e., *C. vulgaris*, *E. cinerea*, *E. umbellata*) and a balanced mixture of all three species on the three main stages of the parasite cycle of *T. cirucmcincta* (i.e., egg, infective larvae and adult worms), were measured using an egg hatching assay (EHA), a larval exsheathment inhibition assay (LEIA) and an adult motility inhibition assay (AMIA). For *H. contortus*, only EHA and LEIA were performed.

2.2. Heather extracts

Non-purified extracts of total phenolics were obtained following the procedures described by Makkar et al. (1993). First, pigments and fats were removed from dried plant material using diethyl ether containing 1% acetic acid. Total phenols were then extracted with 70% aqueous acetone. This extract contains mainly tannins but also other phenolic compounds.

2.3. Parasites

T. circumcincta eggs, larvae and adults for EHA, LEIA and AMIA were obtained from two donor goats experimentally infected *per os* with 6,000 infective larvae (goat strain). Eggs and larvae of *H. contortus* for EHA and LEIA were obtained from one donor goat experimentally infected with 3,000 infective larvae (goat strain). For LEIA, batches of 2- to 3-month-old larvae were used in the assays. For AMIA, four weeks after infection, donor goats infected with *T. circumcincta* were euthanised and immediately after death, the abomasa were collected, opened, briefly washed and placed in a Baermann apparatus with saline at 37°C. After 2 h, worms that had migrated to the saline were collected.

2.4. Egg hatching assay

The method was based on a modification of the egg hatch assay performed to measure anthelmintic resistance (WAAVP recommendations, Coles et al., 1992). Eggs were extracted from fresh faeces by repeated centrifugations and washings. Briefly, a water suspension of faeces was filtered through a mesh (150 μm pore size) and transferred into 15 mL centrifuge tubes. The suspension was centrifuged (4,500 rpm, 5 min, 20ºC), removing the supernatant and adding tap water, three times. After the third centrifugation, the supernatant was removed, replaced with saturated salt solution and centrifuged (4,500 rpm, 5 min, 20ºC) two times. The eggs (located on the top of the saline solution, approximately 1 mL) were collected and kept in a plastic tube (50 mL). The tube was filled with Phosphate Buffered Saline (PBS; 0.1 M phosphate, 0.05 M NaCl, pH=7.2) and the egg solution was centrifuged, the supernatant removed, and the tube filled again with PBS in order to prevent any contact of the eggs with the saline solution. Then, the eggs were distributed in 24-well multiwell plates at a density of 100 eggs per well. Increasing concentrations of plant extracts (75, 150, 300, 600 and 1,200 µg dry matter/mL) were obtained from lyophilised extracts dissolved in PBS and then added to each well. Each concentration was tested using four replicates. Eggs were incubated for 48 h at 25°C. Thereafter, the number of larvae present per well was counted, and the percentage hatched was determined as the ratio between the number of larvae to the number of eggs deposited per well. A mean percentage of hatching was calculated for each concentration of each plant extracts.

2.5. Larval artificial exsheathment assay

Firstly, 1,500 ensheathed larvae were incubated for 3 h at 20°C with one of the four heather extracts at concentrations of 150, 300, 600 and 1,200 µg/mL diluted in PBS. PBS was used to avoid interference from any non-specific effect due to a pH change. After incubation, the ensheathed larvae were washed three times in PBS. After this first step of incubation with plant extracts, the larvae were submitted to artificial exsheathment by contact with a solution of sodium hypochlorite (2% w/v) and sodium chloride (16.5% w/v) diluted 1 to 150 (for *T. circumcincta*) or 1 to 300 (for *H. contortus*) in PBS. The kinetics of larval exsheathment were then measured by microscopic observation for each experimental treatment; the identification of exsheathed larvae was performed by regular examination at x20 magnification at 0, 10, 20, 30, 40, 50 and 60 min (for *T. circumcincta*) and 0, 20, 40 and 60 min (for *H. contortus*) after contact with the exsheathment solution. Four replicates were run for each extract to examine the changes in proportion of exsheathed larvae with time.

2.6. Adult motility inhibition assay

The adult *T. circumcincta* worms collected were immediately placed in a 48-well multiwell plate at a concentration of two nematodes per well. The plates were maintained at 37°C throughout. Worms were first washed for 1 h in PBS with penicillin and streptomycin at concentration of 4%. Thereafter, 1 mL of the different concentrations (75, 150, 300, 600 and 1,200 µg/mL) of heather extracts diluted in PBS (with penicillin and streptomycin 4%) was added to the wells. Levamisole (at concentrations of 0.125%, 0.25%, 0.5% and 1%) was used as an anthelmintic control, and PBS controls were included on each plate. For each treatment, measurements were made on six replicates (12 worms) per plate. The supernatant was changed every 24 hours. The mobility of adult worms was noted by careful observation under a stereomicroscope at x40 magnification after 0, 19, 24, 43, 48, 67 and 72 h. At each time, a motility index was calculated as the ratio between the number of mobile worms and the total number of worms.

2.7. Statistical analysis

For EHA, the significance of the differences of the mean proportions of unhatched eggs among treatments was assessed using the general linear models (GLM) procedures with the Systat 9 software (SPSS Ltd.). For LEIA, the GLM statistical test was performed to determine the difference in the mean percentage of exsheathment rates between the control and different dose groups across time using the SAS statistic package (SAS, 1999). The extract concentration required to inhibit 50% of egg hatching or 50% of the larvae exsheathment rate (EC_{50}) was calculated using the POLO plus statistical program. In AMIA, the number of immobile worms for each treatment (plant extract and dose) was recorded over time. The survival analysis was examined with the non-parametric stratified Cox regression test using the Systat 9 software. For the three *in vitro* assays, the dose response relationship was

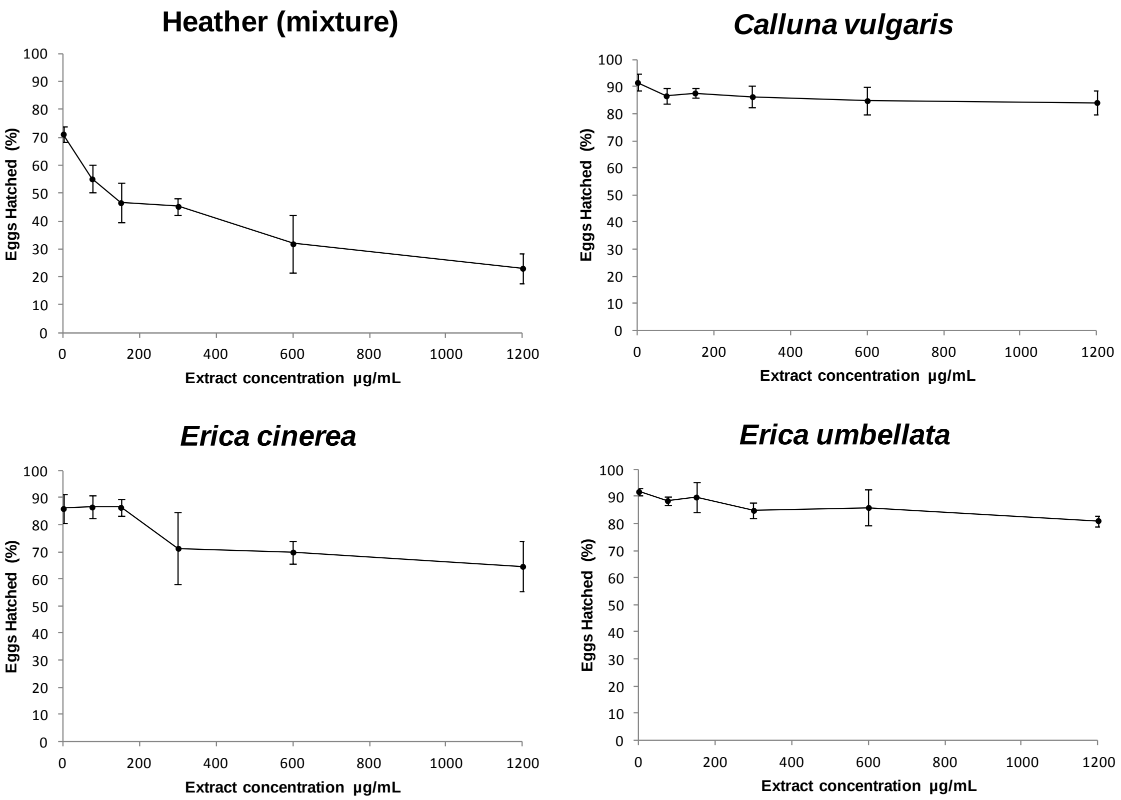

Figure 1. The effect of different concentrations of heather extracts (*Calluna vulgaris*, *Erica cinerea*, *Erica umbellata* and a mixture of all three) on the proportion of *T. circumcincta* eggs hatched *in vitro*. Each point

determined considering the statistical level of significance to be P<0.05.

3. Results

3.1. Egg hatching assay

The mean *T. circumcincta* hatching rate measured in the PBS control was 85.2%. Incubation with *E. cinerea*, *E. umbellata* and mixed heather extracts caused a significant (P<0.01) dose-dependent effect on *T. circumcincta* egg hatching (Figure 1). The mixture of heather extracts was a much more effective inhibitor of *T. circumcincta* egg hatching, considering that the EC_{50} for the mixed extracts was evaluated to be close to 450 µg/mL, whereas a 50% reduction of hatching was not reached with the extracts of each separate species, thus making impossible to calculate EC_{50}.

The mean *H. contortus* hatching rate measured in the PBS control was 92.6%. *E. cinerea* extracts significantly (P<0.01) inhibited *H. contortus* egg hatching, and *C. vulgaris* and *E. umbellata* extracts showed a dose-dependent effect (Figure 2). The extract concentrations assayed for *H. contortus* did not inhibit more than 50% of egg hatching.

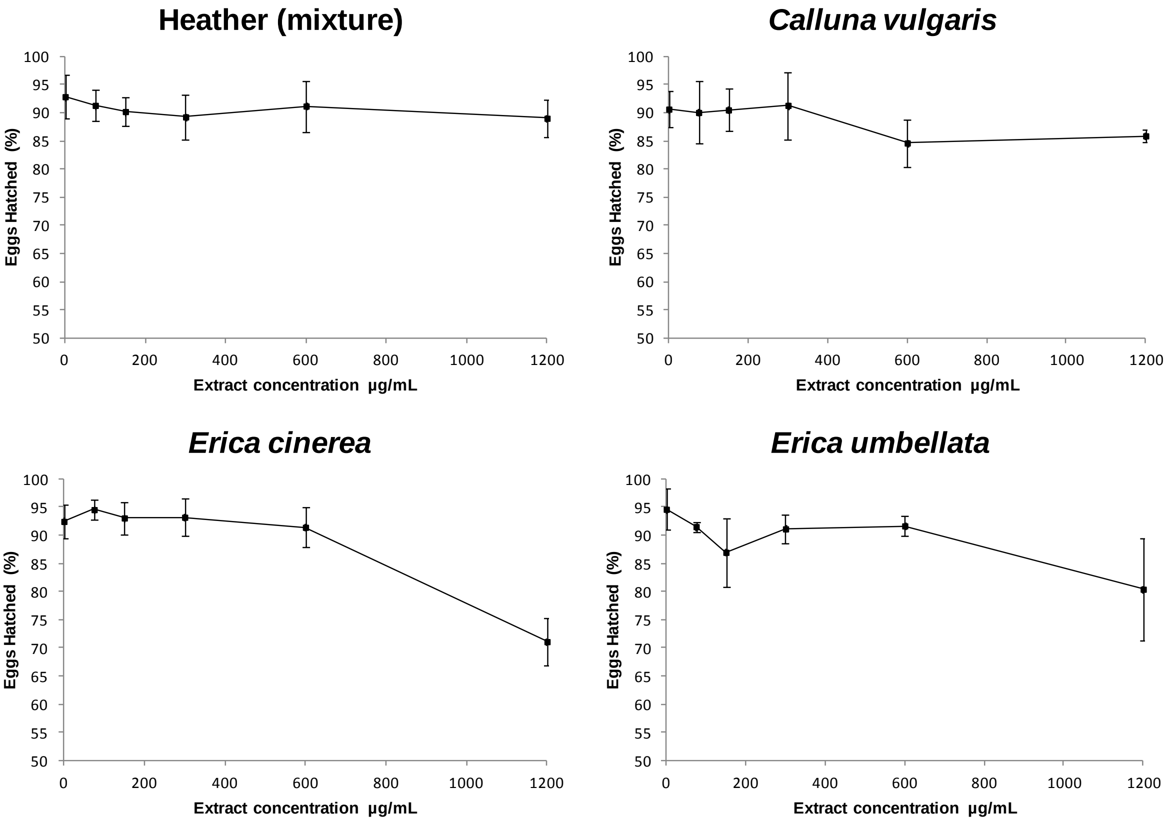

Figure 2. The effect of different concentrations of heather extracts (*Calluna vulgaris*, *Erica cinerea*, *Erica umbellata* and a mixture of all three) on the proportion of *H. contortus* eggs hatched *in vitro*. Each point represents the mean (±S.D.) of four replicates per extract and concentration.

3.2. Larval artificial exsheathment assay

In the PBS control, more than 90% of the *T. circumcincta* larvae were exsheathed 40 minutes after the deposition of the sodium chloride solution. Significant (P<0.001) differences were observed in the exsheathment process between the *T. circumcincta* larvae in PBS and those incubated in all heather extracts (Figure 3). The *T. circumcincta* larvae exsheathment was delayed, and the effect was dose-dependent**.**

More than 95% of *H. contortus* larvae in the PBS control were exsheathed 60 minutes after the deposition of sodium chloride solution. Significant (P<0.001) differences were found at all concentrations tested (150, 300, 600 and 1,200 µg/mL) in the exsheathment process between the *H. contortus* larvae in PBS and those incubated in all heather extracts (Figure 4). *H. contortus* larvae exsheathment was completely inhibited when larvae were incubated with the highest concentrations. Except for the *E. cinerea* extract, the EC_{50} values of all extracts were lower in *H. contortus* than in *T. circumcincta* (Table 1).

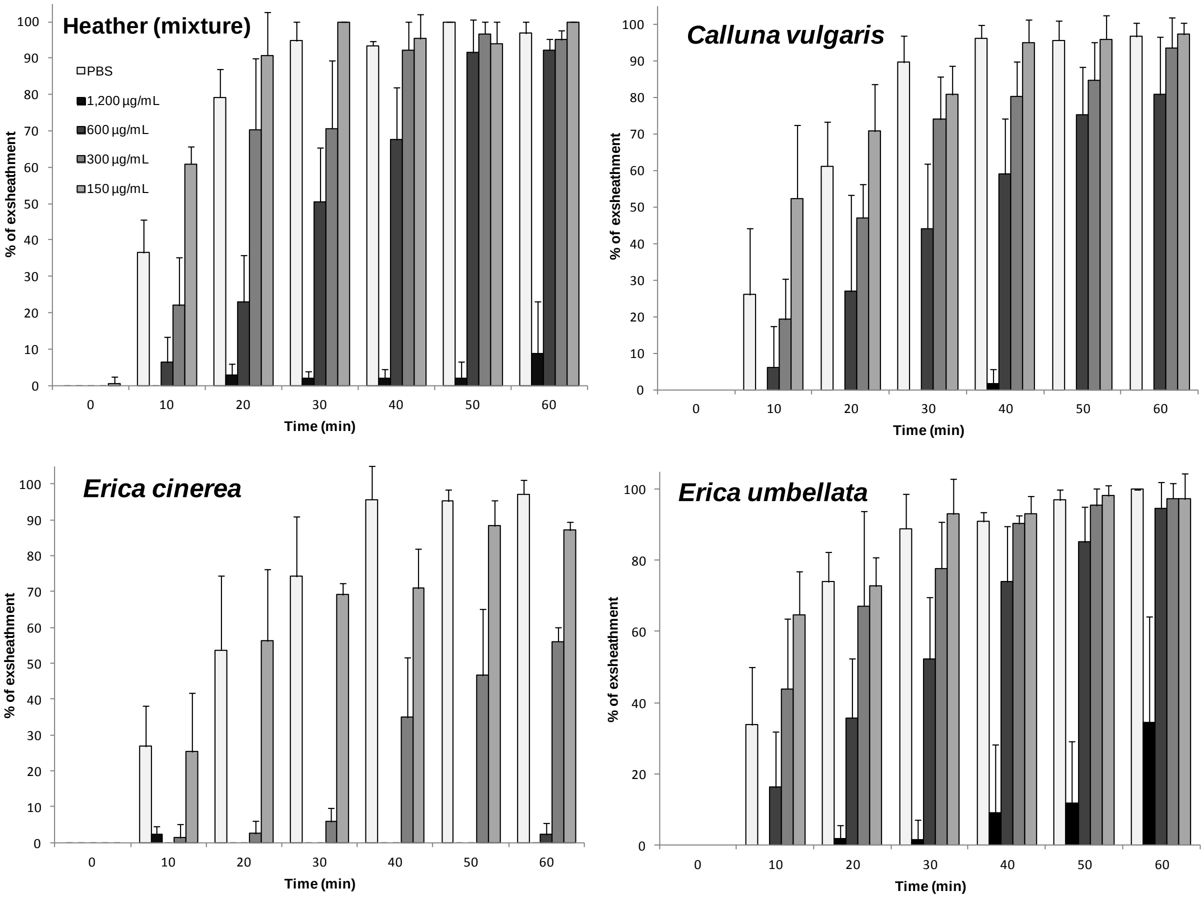

Figure 3. The effect of different concentrations of heather extracts on *T. circumcincta* third-stage larvae after a 3-h incubation and artificial *in vitro* exsheathment, provoked by the addition of a 2% solution of hypochloride. Each time point represents the mean (±S.D.) of four replicates.

3.3. Adult motility inhibition assay

A significant (P<0.05) reduction in worm motility was observed when *T. circumcincta* adult worms were incubated with the highest (1,200 µg/mL) heather extract concentration (Figure 5) for all heather species after 19 h of exposure, but *E. cinerea* (43 h). However, differences in the results were observed between the different extracts at the lowest concentrations. The effects of the mixed extracts were significant at all concentrations. Significant effects were also observed for *C. vulgaris* and *E. umbellata* at 600 µg/mL.

4. Discussion

The general objective of the present work was to evaluate the *in vitro* effects of phenolic extracts from some heather species on the different development stages (e.g., eggs, infective larvae and adult worms) of the abomasal nematode *T. circumcincta*. In a previous report, the reduction in faecal *T. circumcincta* egg counts and worm burden were shown in goats that were naturally infected with gastrointestinal nematodes and fed heather (Osoro et al, 2009). Recently, the reduction in the establishment of infective larvae and the fertility of female nematode parasites were

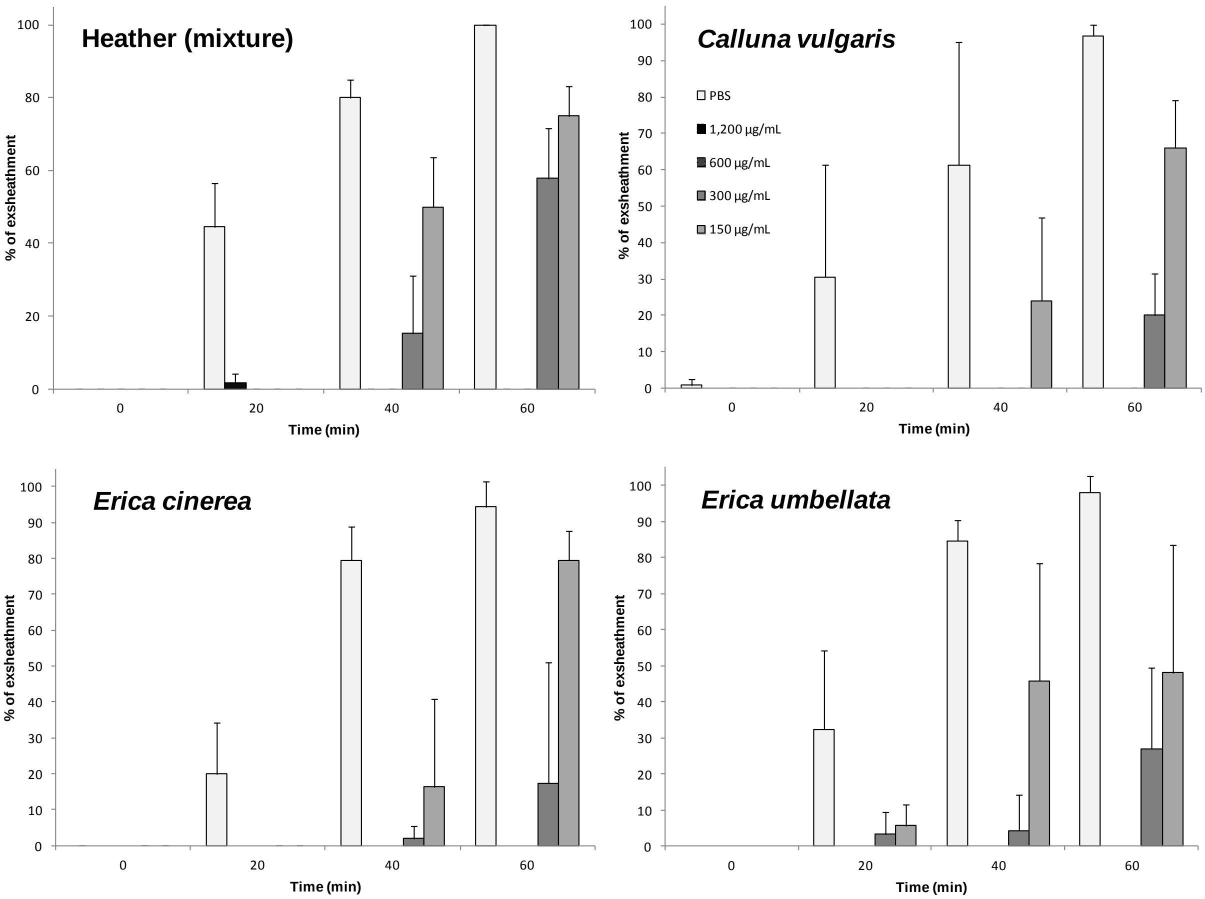

Figure 4. The effect of different concentrations of heather extracts on *H. contortus* third-stage larvae after a 3-h incubation and artificial *in vitro* exsheathment, provoked by the addition of a 2% solution of hypochloride. Each time point represents the mean (±S.D.) of four replicates.

confirmed in controlled trials, which included goats experimentally infected with *T. circumcincta* and supplemented with heather (Moreno-Gonzalo et al., 2013). The effects on the eggs and infective larvae of another important abomasal nematode, *H. contortus*, were also measured after incubation with the same heather extracts.

The anthelmintic effects of plants on nematodes could vary depending on the plant species or stage or the nematode species and the parasitic stage involved (Hoste et al., 2006). Therefore, it is important to test the local plants or forages with the most pathogenic or prevalent parasites species of each area to apply this knowledge to practical conditions. However, testing a bioactive plant with a subset of parasite species would allow us to compare and quantify its potential anthelmintic action. *T. circumcincta* is the most prevalent gastrointestinal nematode found in goats in temperate dry areas, such as Central (Valcárcel et al., 1999) and Northern (Osoro et al., 2007) Spain. *H. contortus* has been used as a model to test the *in vitro* anthelmintic properties of numerous plants forages or their extracts (Kotze et al., 2009; Manolaraki et al.,

Table 1. The extract concentration required to inhibit 50% of infective larvae exsheathment (EC_{50}) and the lower and upper 90% limits for the different total phenolic heather (i.e., *Calluna vulgaris*, *Erica cinerea*, *Erica umbellata* and a mixture of all three) extracts for the larval exsheathment inhibition assay.

	EC_{50} (µg/mL) *	Lower limit 90% (µg/mL)	Upper limit 90% (µg/mL)
T. circumcincta			
Calluna vulgaris	606.3	515.9	687.8
Erica cinerea	213.2	191.1	235,6
Erica umbellata	772.1	667.5	873.9
Mixture	695.7	632.8	778.9
H. contortus			
Calluna vulgaris	188.6	167.1	208.5
Erica cinerea	222.3	178.2	259.9
Erica umbellata	179.4	117.8	225.6
Mixture	253.5	219.4	290.1

* EC_{50} values were calculated at 40 and 60 minutes, respectively, for *T. circumcincta* and *H. contortus*. (All *T. circumcincta* and *H. contortus* third-stage larvae were exsheathed at 40 and 60 minutes, respectively, in the control groups).

2010; Katiki et al., 2011; Kamaraj et al., 2011; Oliveira et al., 2009, 2011; Cala et al., 2012; Marie-Magdeleine et al., 2009, 2010; Hussain et al., 2011), but studies on *T. circumcincta* are scarce (Molan et al., 2004, Molan and Faraj., 2010; Paolini et al., 2004; Brunet et al., 2008).

The first specific objective of our study was to assess the interference of phenolic extracts from heather on *T. circumcincta* and *H. contortus* egg hatching. In *T. circumcincta*, the incubation of eggs with *E. cinerea*, *E. umbellata* and mixed heather extracts significantly reduced the egg hatching rate. However, the information on the *in vitro* effects of different plant extracts on *T. circumcincta* egg hatching is scarce. Molan and Faraj (2010) tested a number of plant extracts (i.e., *Lotus pedunculatus*, *Lotus corniculatus*, *Dorycnium pentaphyllum*, *Dorycnium rectum*, *Rumex obtusifolius*) showing a reduction in *T. circumcincta* egg hatching at 900 µg/mL. Al-Rofaai et al. (2012) tested *Azadirachta indica* and *Manihot esculenta* extracts and found significant reductions of *T. circumcincta* egg hatching only at very high concentrations (>12.5 mg/mL). Interestingly, our results showed an important effect of mixed heather extracts (EC_{50} = 450 µg/mL) but little to no effect of each heather species applied separately. Although the trials should be repeated to confirm the observed differences, these results could suggest a synergic action of polyphenolic compounds present in heather species. This fact could have important consequences in field conditions where goats browse on different heather species but further information is needed, especially in *in vivo* conditions.

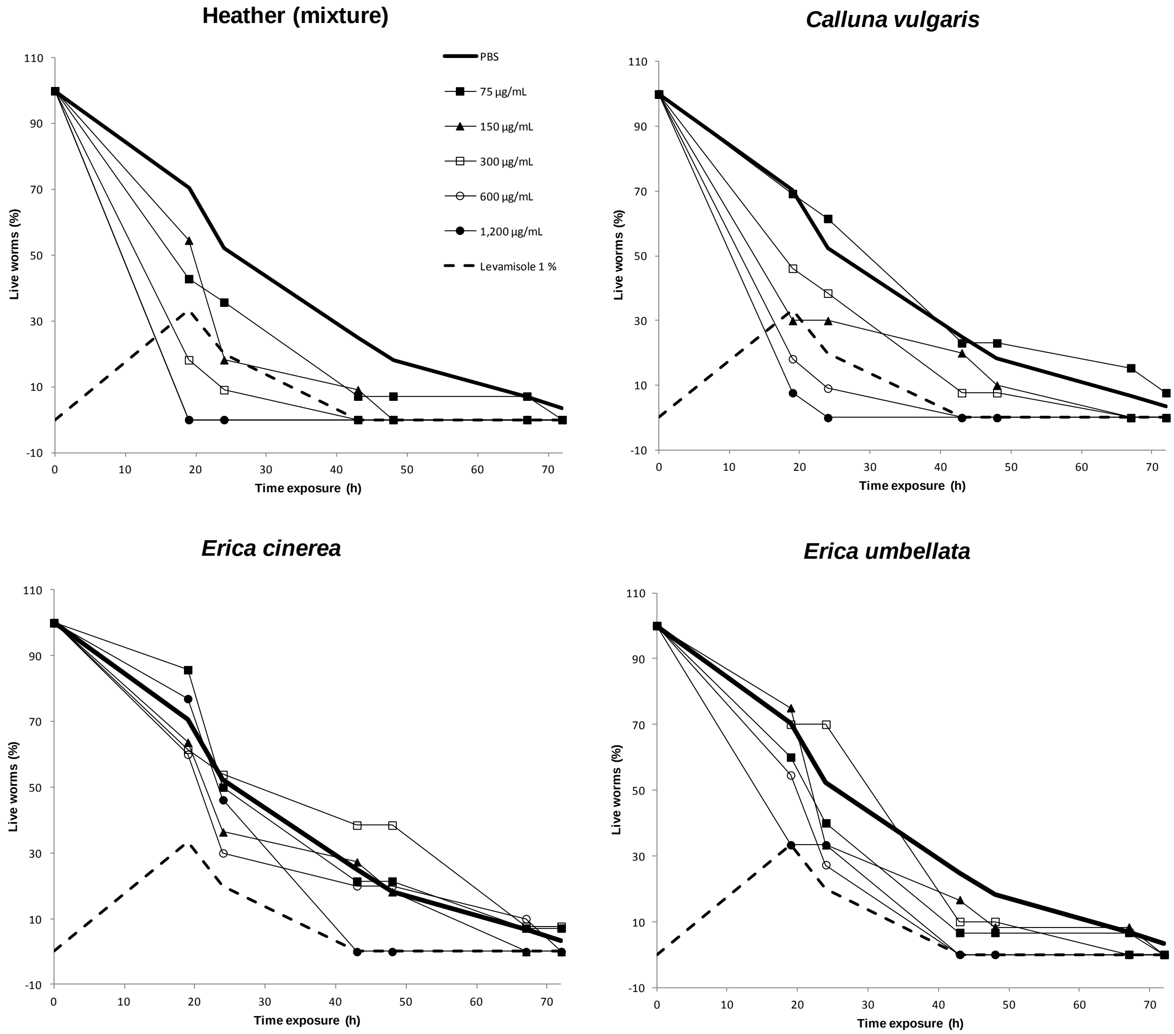

Figure 5. Anthelmintic activity over time of heather extracts compared with positive (levamisole 1%) and negative (Phosphate Buffered Saline, PBS) controls on *T. circumcincta* adult worms, expressed as the percentage of live worms (mobile worms) among the total exposed worms.

Only the *E. cinerea* extracts significantly reduced *H. contortus* egg hatching. Other studies using the same assay and parasite but with other plant extracts using similar (Hounzangbe-Adote et al., 2005: *Zanthoxylum zanthoxyloides*, *Newbouldia laevis*, *Morinda lucida* and *Carica papaya*; Eguale et al., 2007: *Hedera helix*; Oliveira et al., 2009: *Cocos nucifera*; Oliveira et al., 2011: *Myracrodruon urundeuva*) or higher (Assis et al., 2003: *Spigelia anthelmia*; Maciel et al., 2006: *Melia azedarach*; Costa et al., 2008: *Azadirachta indica*; Bachaya et al., 2009: *Ziziphus nummularia* (bark) and *Acacia nilotica* (fruit); Monteiro et al., 2011 *Jatropha curcas* (seeds)) concentrations showed significant effects on the egg hatching rate. However, Alawa et al. (2003) found no effect when *H. contortus* eggs were incubated with *Vernonia amygdalina*, but a reduction was observed with *Annona senegalensis* extracts at 7.1 mg/mL. In the egg hatching assay, the mixed heather extract seems to be less effective than each species extract separately for *H. contortus*, but the effect on *T. circumcincta* seems to be the opposite. The explanation could be related with the composition of each extract, which could be different. The extract including mixed heather species could contain all the active compounds presents in each one of the other three species extracts investigated. It is known that different condensed tannin monomers have different activities and the degree of polymerization or the number of free hydroxy groups is a key factor in the interactions with parasites (Brunet and Hoste, 2006). On the other hand, a synergic or antagonistic action of components in mixed heather extract is also possible (Copani et al., 2012). Furthermore, differences on the anthelmintic effect of tannins due to the plant or to the nematode species involved have been observed (Hoste et al., 2006).

The second specific objective was to study the interference of the heather extracts on the exsheathment of *T. circumcincta* and *H. contortus* infective larvae. The results showed that the four extracts blocked the exsheathment process at the highest concentration (1,200 µg/mL) tested but that the lowest concentration (150 µg/mL) was not sufficient to provoke any effect on the exsheathment. Molan et al. (2004) showed a decrease in *T. circumcincta* infective larvae motility with *Camellia sinensis* extracts using a larval migration inhibition assay. A reduction in *T. circumcincta* infective larvae motility with *A. indica* and *M. esculenta* extracts using a larval paralysis assay was also reported (Al-Rofaai et al., 2012). The heather extracts blocked (at 1,200 and 600 µg/mL) or delayed (at 300 and 150 µg/mL) larvae exsheathment when *H. contortus* was tested. Bahuaud et al. (2006) obtained similar results when testing another heather species (*Erica erigena*). Azando el al. (2011) also observed a high level of *H. contortus* larval exsheathment inhibition with *Newbouldia laevis* and *Zanthoxylum zanthoxyloides* extracts.

For trichostrongile nematodes the exsheathment of infective larvae is a crucial step in the life-cycle since it represents the transition from the free-living to the parasitic stages (Hertzberg et al., 2002). Therefore, a partial or total inhibition of infective larvae exsheathment caused by heather consumption could contribute to reducing the worm burden. This effect of heather extracts on the exsheathment observed in *T. circumcincta* infective larvae could explain the significant reduction in the establishment rate observed six days post-infection in goats experimentally infected with *T. circumcincta* and supplemented with heather (Moreno-Gonzalo et al., 2013).

The third specific objective of the current study was to investigate the possible effect of heather phenolic extracts on the viability of *T. circumcincta* adult worms. The incubation with heather phenolic extracts at high concentrations (>600 µg/mL) reduced the viability of adult *T. circumcincta* worms. This effect could explain the reduction in worm burden and faecal egg output in grazing goats consuming heather that were naturally infected with *T. circumcincta* and other trichostrongyles (Osoro et al., 2009). The early decrease in faecal egg counts caused by the reduced fertility of female worms when experimentally infected goats started heather consumption suggests a direct effect of heather on *T. circumcincta* adult worms (Moreno-Gonzalo et al., 2013). As far as we know, the only previous report on the *in vitro* effects of plant extracts on *T. circumcincta* adult worms using AMIA tested the effects of *Rubus fruticosus*, *Quercus robur*, *Corylus avellana* and *Onobrychis viciifolia* extracts and showed a reduction in worm motility (Paolini et al., 2004).

Conclusions

The main result of this work is that phenolic extracts from heather showed anthelmintic activity on *T. circumcincta* eggs, larvae and adult worms and on *H. contortus* egg hatching and larvae exsheathment. The infective larvae exsheathment assay seems to be the most sensitive method to measure the action of phenolic compounds included in heather. The results confirm the potential use of heather to control gastrointestinal nematode infections in small ruminants infected with abomasal species such as *T. circumcincta* or *H. contortus*.

Conflict of interest

None of the authors has any financial or personal relationship that could inappropriately influence or bias the content of the paper.

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References

Ademola, I.O., Akanbi, A.I., Idowu, S.O., 2005. Comparative nematocidal activity of chromatographic fractions of *Leucaena leucocephala* seed against gastrointestinal sheep nematodes. Pharm. Biol. 43, 599-604.

Akkari, H., Ben Salem, H., Gharbi, M., Abidi, S., Darghouth, M.A., 2008. Feeding *Acacia cyanophylla* Lindl. foliage to Barbarine lambs with or without PEG: Effect on the excretion of gastro-intestinal nematode eggs. Anim. Feed Sci. Technol. 147, 182-192.

Alawa, C.B.I., Adamu, A.M., Gefu, J.O., Ajanusi, O.J., Abdu, P.A., Chiezey, N.P., Alawa, J.N., Bowman, D.D., 2003. *In vitro* screening of two Nigerian medicinal plants (*Vernonia amygdalina* and *Annona senegalensis*) for anthelmintic activity. Vet. Parasitol. 113, 73-81.

Al-Rofaai, A., Rahman, W.A., Sulaiman, S.F., Yahaya, Z.S., 2012. *In vitro* activity of neem (*Azadirachta indica*) and cassava (*Manihot esculenta*) on three pre-parasitic stages of susceptible and resistant strains of *Teladorsagia* (*Ostertagia*) *circumcincta*. Vet. Parasitol. 188, 85-92.

Assis, L.M., Bevilaqua, C.M.L., Morais, S.M., Vieira, L.S., Costa, C.T.C., Souza, J.A.L., 2003. Ovicidal and larvicidal activity *in vitro* of *Spigelia anthelmia* Linn. extracts on *Haemonchus contortus*. Vet. Parasitol. 117, 43-49.

Azando, E.V.B., Hounzangbe-Adote, M.S., Olounlade, P.A., Brunet, S., Fabre, N., Valentin, A., Hoste, H., 2011. Involvement of tannins and flavonoids in the *in vitro* effects of *Newbouldia laevis* and *Zanthoxylum zanthoxyloides* extracts on the exsheathment of third-stage infective larvae of gastrointestinal nematodes. Vet. Parasitol. 180, 292-297.

Bachaya, H.A., Iqbal, Z., Khan, M.N., Sindhu, Z.U.D., Jabbar, A., 2009. Anthelmintic activity of *Ziziphus nummularia* (bark) and *Acacia nilotica* (fruit) against Trichostrongylid nematodes of sheep. J. Ethnopharmacol. 123, 325-329.

Bahuaud, D., De Montellano, C.M.O., Chauveau , S., Prevot, F., Torres-Acosta, F., Fouraste, I., Hoste, H., 2006. Effects of four tanniferous plant extracts on the *in vitro* exsheathment of third-stage larvae of parasitic nematodes. Parasitology 132, 545-554.

Barrau, E., Fabre, N., Fouraste, I., Hoste, H., 2005. Effect of bioactive compounds from Sainfoin (*Onobrychis viciifolia* Scop.) on the *in vitro* larval migration of *Haemonchus contortus*: role of tannins and flavonol glycosides. Parasitology 131, 531-538.

Brunet, S., Hoste, H., 2006. Monomers of condensed tannins affect the larval exsheathment of parasitic nematodes of ruminants. J. Agric. Food Chem. 54, 7481-7487.

Brunet, S., Jackson, F., Hoste, H., 2008. Effects of sainfoin (*Onobrychis viciifolia*) extract and monomers of condensed tannins on the association of abomasal nematode larvae with fundic explants. Int. J. Parasitol. 38, 783-790.

Cala, A.C., Chagas, A.C.S., Oliveira, M.C.S., Matos, A.P., Borges, L.M.F., Sousa, L.A.D., Souza, F.A., Oliveira, G.P., 2012. *In vitro* anthelmintic effect of *Melia azedarach* L. and *Trichilia classenii* C. against sheep gastrointestinal nematodes. Exp. Parasitol. 130, 98-102.

Coles, G.C., Bauer, C., Borgsteede, F., Geerts, S., Klei, T.R., Taylor, M.A., Waller, P.J., 1992. World Association for Advancement in Veterinary Parasitology (WAAVP) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. Vet. Parasitol. 44, 35-43.

Copani, G., Hall, J.O., Miller, J., Priolo, A., Villalba, J.J., 2012. Plant secondary compounds as complementary resources: are they always complementary? Oecologia, doi 10.1007/s00442-012-2551-1.

Costa, C.T.C., Bevilaqua, C.M.L., Camurca-Vasconcelos, A.L.F., Maciel, M.V., Morais, S.M., Castro, C.M.S., Braga, R.R., Oliveira, L.M.B., 2008. *In vitro* ovicidal and larvicidal activity of *Azadirachta indica* extracts on *Haemonchus contortus*. Small Rumin. Res. 74, 284-287.

Eguale, T., Tilahun, G., Debella, A., Feleke, A., Makonnen, E., 2007. *Haemonchus contortus*: *In vitro* and *in vivo* anthelmintic activity of aqueous and hydro-alcoholic extracts of *Hedera helix*. Exp. Parasitol. 116, 340-345.

Frutos, P., Hervás, G., Celaya, R., Moreno-Gonzalo, J., Rodríguez, A.B., García, U., Ferre, I., Ortega-Mora, L.M., Osoro, K., 2011. Effects of the use of heather as anthelmintic in goats infected with *Trichostrongylus colubriformis* on ruminal fermentation and digestibility. Opt. Méd. 99, 321-327.

Hertzberg, H., Huwyler, U., Kohler, L., Rehbein, S., Wanner, M., 2002. Kinetics of exsheathment of infective ovine and bovine strongylid larvae *in vivo* and *in vitro*. Parasitology 125, 65-70.

Hoste, H., Jackson, F., Athanasiadou, S., Thamsborg, S.M., Hoskin, S.O., 2006. The effects of tannin-rich plants on parasitic nematodes in ruminants. Trends Parasitol. 22, 253–261.

Hoste, H., Torres-Acosta, J.F.J., 2011. Non chemical control of helminths in ruminants: Adapting solutions for changing worms in a changing world. Vet. Parasitol. 180, 144-154.

Hoste, H., Martinez-Ortiz-De-Montellano, C., Manolaraki, F., Brunet, S., Ojeda-Robertos, N., Fourquaux, I., Torres-Acosta, J.F.J., Sandoval-Castro, C.A., 2012. Direct and indirect effects of bioactive tannin-rich tropical and temperate legumes against nematode infections. Vet. Parasitol. 186, 18-27.

Hounzangbe-Adote, M.S., Paolini, V., Fouraste, I. Moutairou, K., Hoste, H., 2005. *In vitro* effects of four tropical plants on three life-cycle stages of the parasitic nematode *Haemonchus contortus*. Res. Vet. Sci. 78, 155-160.

Hussain, A., Khan, M.N., Iqbal, Z., Sajid, M.S., Khan, M.K., 2011. Anthelmintic activity of *Trianthema portulacastrum* L. and *Musa paradisiaca* L. against gastrointestinal nematodes of sheep. Vet. Parasitol. 179, 92-99.

Kabasa, J.D., Opuda-Asibo, J., ter Meulen, U., 2000. The effect of oral administration of polyethylene glycol on faecal helminth egg counts in pregnant goats grazed on browse containing condensed tannins. Trop. Anim. Health Prod., 32, 73-86.

Kamaraj, C., Rahuman, A.A., 2010. Efficacy of anthelmintic properties of medicinal plant extracts against *Haemonchus contortus*. Res. Vet. Sci. 10, 1-5.

Kaplan, R.M., 2004. Drug resistance in nematodes of veterinary importance: a status report. Trends Parasitol. 20, 477-481.

Katiki, L.M., Chagas, A.C.S., Bizzo, H.R., Ferreira, J.F.S., Amarante, A.F.T., 2011. Anthelmintic activity of *Cymbopogon martinii*, *Cymbopogon schoenanthus* and *Mentha piperita* essential oils evaluated in four different *in vitro* tests. Vet. Parasitol. 183, 103-108.

Kotze, A.C., O´Grady, J.O., Emms, J., Toovey, A.F., Hughes, S., Jessop, P., Bennell, M.R., Vercoe, P.E., Revell, D.K., 2009. Exploiting the anthelmintic properties of Australian native shrubs with respect to their potential role in livestock grazing systems. Parasitology 136, 1065-1080.

Landau, S., Azaizeh, H., Muklada, H., Glasser, T., Ungar, E.D., Baram, H., Abbas, N., Markovics, A., 2010. Anthelmintic activity of Pistacia lentiscus foliage in two Middle Eastern breeds of goats differing in their propensity to consume tannin-rich browse. Vet. Parasitol. 173, 280-286.

Maciel, M.V., Morais, S.M., Bevilaqua, C.M.L., Camurça-Vasconcelos, A.L.F., Costa, C.T.C., Castro, C.M.S., 2006. Ovicidal and larvicidal activity of *Melia azedarach* extracts on *Haemonchus contortus*. Vet. Parasitol. 140, 98-104.

Makkar, H.P.S., Blümmel, M., Borowy, N.K., Becker, K., 1993. Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. J. Sci. Food Agric. 61, 161-165.

Manolaraki, F., Sotiraki, S., Stefanakis, A., Skampardonis, V., Volanis, M., Hoste, H., 2010. Anthelmintic activity of some Mediterranean browse plants against parasitic nematodes. Parasitology 137, 685-696.

Marie-Magdeleine, C., Hoste, H., Mahieu, M., Varo, H., Archimede, H., 2009. *In vitro* effects of *Cucurbita moshata* seed extracts on *Haemonchus contortus*. Vet. Parasitol. 161, 99-105.

Marie-Magdeleine, C., Udino, L., Philibert, L., Bocagea, B., Archimede, H., 2010. *In vitro* effects of Cassava (*Manihot esculenta*) leaf extracts on four development stages of *Haemonchus contortus*. Vet. Parasitol. 173, 85-92.

Molan, A.L., Sivakumaran, S., Spencer, P.A., Meagher, L.P., 2004. Green tea flavan-3-ols and oligomeric proanthocyanidins inhibit the motility of infective larvae of *Teladorsagia circumcincta* and *Trichostrongylus colubriformis in vitro*. Res. Vet. Sci. 77, 239-243.

Molan, A.L., Faraj, A.M., 2010.The effects of condensed tannins extracted from different plant species on egg hatching and larval development of *Teladorsagia circumcincta* (Nematoda: Trichostrongylidae). Folia Parasitol. (Praha) 57, 62-68.

Monteiro, M.V.B., Bevilaqua, C.M.L., Morais, S.M., Machado, L.K.A., Camurça-Vasconcelos, A.L.F., Campello, C.C., Ribeiro, W.L.C., Mesquita, M.D.A., 2011. Anthelmintic activity of *Jatropha curcas* L. seeds on *Haemonchus contortus*. Vet. Parasitol. 182, 259-263.

Moreno-Gonzalo, J., Ferre, I., Celaya, R., Frutos, P., Ferreira, L.M.M., Hervás, G., García, U., Ortega-Mora L.M., Osoro, K., 2012. Potential use of heather to control gastrointestinal nematodes in goats. Small Rumin. Res. 103, 60–68.

Moreno-Gonzalo, J., Osoro, K., García, U., Frutos, P., Celaya, R., Ferreira, L.M.M., Ortega-Mora, L.M., Ferre, I, 2013. Effect of the consumption of heather on incoming larvae and established population of *Teladorsagia circumcincta* in experimentally infected Cashmere goats. Vet. Parasitol., doi 10.1016/j.vetpar.2013.02.005.

Oliveira, L.M.B., Bevilaqua, C.M.L., Costa, C.T.C., Macedo, I.T.F., Barros, R.S., Rodrigues, A.C.M., Camurça-Vasconcelos, A.L.F., Morais, S.M., Lima, Y.C., Vieira, L.S., Navarro, A.M.C., 2009. Anthelmintic activity of *Cocos nucifiera* L. against sheep gastrointestinal nematodes. Vet. Parasitol. 159, 55-59.

Oliveira, L.M.B., Bevilaqua, C.M.L., Macedo, I.T.F., Morais, S.M., Machado, L.K.A., Campello, C.C., Mesquita, M.D., 2011. Effects of *Myracrodruon urundeuva* extracts on egg hatching and larval exsheathment of *Haemonchus contortus*. Parasitol. Res. 109, 893-898.

Osoro, K., Mateos-Sanz, A., Frutos, P., García, U., Ortega-Mora, L.M., Ferreira, L.M.M., Celaya, R., Ferre, I., 2007. Anthelmintic and nutritional effects of heather supplementation on Cashmere goats grazing perennial ryegrass-white clover pastures. J. Anim. Sci. 85, 861-870.

Osoro, K., Celaya, R., Moreno-Gonzalo, J., Ferreira, L.M.M., García, U., Frutos, P., Ortega-Mora, L.M., Ferre, I., 2009. Effects of stocking rate and heather supplementation on gastrointestinal nematode infections and host performance in naturally infected Cashmere goats. Rangel. Ecol. Manag. 62, 127-135.

Paolini, V., Fouraste, I., Hoste, H., 2004. *In vitro* effects of three woody plant and sainfoin extracts on 3rd-stage larvae and adult worms of three gastrointestinal nematodes. Parasitology 129, 69-77.

Papachristou, T. G., Dziba, L.E., Provenza, F.D., 2005. Foraging ecology of goats and sheep on wooded rangelands. Small Rumin. Res. 59, 141-156.

Perry, B.D., Randolph, T.F., 1999. Improving the assessment of the economic impact of parasitic diseases and of their control in production animals. Vet. Parasitol. 84, 145-168.

SAS, 1999. SAS/STAT User´s Guide. V8. SAS Institute Inc., Cary, NC, USA.

Valcárcel, F., Romero, C.G., 1999. Prevalence and seasonal pattern of caprine trichostrongyles in a dry area of central Spain. J. Vet. Med. Ser. B-Infect. Dis. Vet. Public Health 46, 673-681.

CAPÍTULO V

Discusión general

Los nematodos gastrointestinales pertenecientes a la superfamilia Trichostrongyloidea que parasitan a los pequeños rumiantes han sido objeto de numerosos estudios en el pasado, conociéndose en la actualidad de forma bastante precisa su ciclo biológico y epidemiología, hecho que ha facilitado el diseño de fármacos eficaces para su control (Zajac, 2006). Sin embargo, las nematodosis gastrointestinales poseen ciertas características que las convierten en procesos de difícil erradicación en explotaciones extensivas o semiextensivas, donde los animales permanecen largos periodos de tiempo en pastoreo. Los nematodos gastrointestinales son capaces de evadir o alterar la respuesta inmune del hospedador (Gasbarre, 1997; Balic et al., 2000). Por ello, pueden afectar a prácticamente cualquier animal, aunque existen grandes diferencias entre individuos, encontrándose animales "resistentes" (*responders*) que albergarían intensidades parasitarias bajas o muy bajas y "no resistentes" (*non-responders*), que albergarían cargas elevadas y causantes de signos clínicos e incluso la muerte del animal (Stear et al., 2007b). Además, al presentar una fase exógena siempre existen parásitos que escapan a los tratamientos antihelmínticos, haciendo casi imposible la total erradicación de estas parasitosis. Por lo tanto, los métodos de control, generalmente, se encaminan a mantener estas parasitosis en niveles lo suficientemente bajos para que no supongan un problema para las explotaciones (Ketsis et al., 2006). Con esta finalidad y para mantener poblaciones parásitas no sometidas a la acción de los fármacos antihelmínticos, retrasándose así el desarrollo de cepas resistentes, se estudia la aplicación de tratamientos selectivos a algunos individuos en base a ciertos criterios, como el recuento de huevos en heces o los indicadores fisiológicos o de producción (Kenyon et al., 2009; Rinaldi y Cringoli, 2012; Besier, 2012). Debe destacarse además que el desarrollo de cepas de nematodos gastrointestinales resistentes a los fármacos antihelmínticos más utilizados en los pequeños rumiantes parece ser especialmente rápido (Várady et al., 2011). En nuestro país, ya se ha descrito la aparición de cepas de nematodos gastrointestinales resistentes en ganado ovino (Álvarez-Sánchez et al., 2006; Díez-Baños et al., 2008; Martínez-Valladares et al., 2013) y caprino (Requejo-Fernández et al., 1997).

Aunque la eficacia mostrada por el consumo de plantas bioactivas (aquellas plantas con compuestos que pueden proteger la salud del animal que las consume) dista mucho de la alcanzada por la administración de fármacos antihelmínticos, que en ausencia de cepas resistentes llega a ser de más del 99% (Hoyt et al., 1992; Chartier, 1995; Chartier y Pors, 2004), su utilización presenta varias ventajas. En primer lugar, no hay presencia de residuos medicamentosos, como ocurre en la carne, leche, heces o la orina de los animales tratados con fármacos sintéticos (Beynon, 2012; Cárceles et al., 2001; McKellar, 1997), evitándose así los periodos de supresión. En segundo, lugar tienen un coste bajo o nulo, tanto en mano de obra para su administración como en el propio producto (la mayoría de ellas son abundantes en la vegetación que pastan los animales).

Se ha demostrado el efecto anticoccidiósico (Hur et al., 2005; Markovics et al., 2012; Saratsis et al., 2012) y antihelmíntico de los taninos condensados así como el de otros metabolitos secundarios (Athanasiadou y Kyriazakis, 2004; Hoste et al., 2006; Athanasiadou et al., 2007), presentes en plantas o forrajes de los que se alimenta el ganado caprino, además de en otras plantas que no forman parte de su dieta habitual. Sin embargo, los resultados obtenidos son muy variables. Aunque la acción de los taninos condensados parece relacionada con la interacción directa de éstos con los parásitos, provocando alteraciones en la hipodermis, formación de vesículas y degeneración o muerte de las células intestinales y musculares (Brunet et al., 2011), o también con la inhibición de procesos enzimáticos de los parásitos, no se ha descrito un mecanismo de acción común y claro en todos los casos (Hoste et al., 2006). Se han observado diferencias en los resultados obtenidos con el mismo parásito utilizando diferentes plantas, con la misma planta y diferentes parásitos, o con la misma planta y parásito dependiendo del estadio parasitario implicado. Incluso, en el caso de experimentos *in vitro* dependiendo del método de extracción, del solvente empleado o de la fracción del extracto utilizada (Ademola et al., 2009; Assis et al., 2003; Molan et al., 2004; Brunet y Hoste 2006; Brunet et al., 2008; Cala et al., 2012). A pesar de que queda mucho por dilucidar en este sentido, parece que la acción de los taninos condensados está muy relacionada con su capacidad para unirse a macromoléculas, en particular proteínas (Mueller-Harvey y McAllan, 1992; Bravo, 1998) y se supone que su acción directa se debe a la interacción con las proteínas del parásito (Paolini et al., 2004; Brunet et al., 2007, 2008; Hoste et al., 2006).

Los taninos condensados han sido considerados durante mucho tiempo sustancias antinutritivas para el ganado, incluyendo también a las cabras (Silanikove et al., 1997; Ben Salem et al., 2003), aunque se ha observado que este hecho depende de varios factores como su estructura o concentración y que pueden ser beneficiosos reduciendo la degradación de proteínas en el rumen o previniendo el timpanismo (Mueller-Harvey, 2006; Min et al., 2003). En la presente tesis se muestra como el consumo de brezo en la proporción ingerida por las cabras (alrededor del 30%) no causó efectos nutritivos adversos, es más, fue beneficioso en ciertos aspectos. Además, redujo la tasa de mortalidad y la pérdida de peso y condición corporal de los animales que lo consumieron.

Las cabras suplementadas con brezo produjeron una menor cantidad de amoníaco en el rumen y una mayor cantidad de ácidos grasos volátiles, principal aporte de energía en los rumiantes, lo que sugeriría una mayor eficiencia en la fermentación ruminal en los animales que consumieron brezo. Al estudiar *in vitro* la fermentación ruminal, incluyendo sustratos procedentes del rumen de cabras que consumieron brezo, se observaron valores ligeramente menores en cuanto a la desaparición de materia seca, digestibilidad, producción de gas y concentración de amoníaco, si lo comparamos con los sustratos de aquellas cabras que consumieron solamente pasto. También se comprobó que la suplementación con brezo redujo la excreción fecal de huevos de nematodos parásitos y esta diferencia se hacía mayor a medida que se incrementaban las excreciones a lo largo de la estación de pastoreo.

Entre los métodos no farmacológicos para el control de las nematodosis gastrointestinales se encuentra el manejo de pastos (Stear et al., 2007a), siendo la presión de pastoreo o carga ganadera un factor determinante.

Aunque se asume que incrementando la presión de pastoreo se incrementa la intensidad parasitaria, la evidencia experimental es escasa (Le Jambre, 1984; Thamsborg et al., 1996) y fundamentalmente proviene de pruebas realizadas con ganado ovino. Nunca antes se habían combinado dos métodos que no incluyeran la utilización de fármacos para el estudio del control de las nematodosis gastrointestinales en ganado caprino, como son el manejo del pastoreo y la utilización de plantas bioactivas. Se pudo comprobar que la excreción fecal de huevos de nematodos gastrointestinales se redujo, tanto en los animales suplementados con brezo como en aquellos sometidos a menor densidad de pastoreo. En general, también se observó siempre una menor intensidad parasitaria en los animales suplementados, aunque en este caso los resultados fueron menos homogéneos. Sin embargo, si valoramos la fecundidad de los parásitos adultos y su desarrollo, no hay una tendencia general. En la mayoría de los casos la fecundidad y el desarrollo fue menor en los vermes procedentes de animales suplementados, pero con importantes excepciones. Esto podría tener varias explicaciones. A medida que aumenta la edad de los vermes, aumenta su longitud y el número de huevos en el útero de las hembras parásitas, lo que podría explicar el mayor tamaño y fecundidad en algunos casos (podrían ser vermes de más edad). Aunque también podría tener influencia la intensidad parasitaria, ya que si un mayor número de parásitos conviven en un mismo hospedador, la mayor densidad podría derivar en un menor desarrollo (Stear y Bishop., 1999). También se ha observado que una mayor intensidad parasitaria en los animales provoca una menor implantación y fecundidad de los nematodos parásitos (Dobson et al, 1990 a,b). Por lo tanto, estos resultados sobre la fecundidad y longitud de los vermes adultos, donde la intensidad parasitaria es muy variable y desconocemos la edad de los parásitos (los animales se están reinfectando continuamente) hay que interpretarlos con precaución, no pudiendo atribuir cualquier diferencia encontrada exclusivamente al consumo o no de brezo.

Por otro lado, se ha observado que dependiendo de la composición de la dieta, la viabilidad y desarrollo de las larvas en las heces puede ser significativamente diferente (Marley et al., 2003) y que los huevos que se encontraban en heces que contenían más taninos, tenían una menor tasa de eclosión (Max, 2010). Aunque este hecho no ha sido estudiado durante la administración de brezo, podría ser otro aspecto interesante para el control de las nematodosis gastrointestinales.

En las infecciones experimentales de cabras con distintas especies de nematodos gastrointestinales se controló la dieta y el consumo de brezo de cada animal, pudiéndose así estudiar el efecto del brezo sobre la implantación de los parásitos (efecto preventivo). En este estudio, utilizando cabras infectadas experimentalmente con *T. colubriformis*, se constató un descenso en la implantación de las larvas infectantes administradas en el grupo que consumió brezo. En este caso desconocemos en qué fase del proceso de implantación se produjo la acción del brezo ya que se realizó un único sacrificio a los 21 días postinfección. En un estudio similar, se observó también la reducción de la implantación de las larvas infectantes de *T. circumcincta* administradas. En este caso, sí se pudo demostrar que el efecto se producía tempranamente durante la primera fase de la implantación de las larvas infectantes, ya que algunos animales se sacrificaron a los cinco días postinfección, no encontrándose diferencias con otros animales sacrificados a los 21 días postinfección. La longitud y fecundidad de las hembras parásitas de *T. circumcincta* en las cabras sacrificadas a los 21 días postinfección fue significativamente menor en aquellas que consumieron brezo. Por lo tanto, el consumo de brezo se asocia a una reducción de la tasa de implantación de las larvas infectantes de *T. colubriformis* y *T. circumcinta* en cabras infectadas experimentalmente. Además, se observó la reducción del desarrollo y fecundidad de *T. circumcinta*.

En los experimentos donde se estudió el efecto curativo del consumo de brezo mediante infecciones experimentales monoespecíficas con ambos nematodos (*T. colubriformis* y *T. circumcincta*) se observó un descenso de la excreción fecal de huevos en el grupo que consumió brezo con respecto al testigo desde los tres o cuatro días de la administración del brezo, lo que hace suponer que existe un efecto directo sobre los parásitos. Se ha demostrado que al mejorar la nutrición de los animales, sobre todo al aumentar la proteína en la dieta, se incrementa la resiliencia del hospedador frente a las nematodosis gastrointestinales (Coop y Kyriazakis, 2001). En este caso, no hay suplementación con brezo como ocurre en los experimentos en pastoreo, sino una sustitución del aporte energético correspondiente por el brezo. Esta razón también apoya la hipótesis de un efecto directo del brezo sobre los parásitos. En el caso de *T. colubriformis,* ese descenso se explicaría por un ligero descenso tanto de la intensidad parasitaria como de la fecundidad de los nematodos. Sin embargo, la intensidad parasitaria de *T. circumcincta* es la misma, pero hay un marcado descenso de la fecundidad de los parásitos. En el experimento en el que se investigó el efecto curativo del consumo de brezo sobre una población patente de *T. colubriformis* en cabras, se incluyó además un grupo de animales que consumió brezo, pero al que también se le administró PEG, un inhibidor de los taninos condensados. Sin embargo, no se observó una reducción del efecto antihelmíntico del brezo, obteniéndose resultados muy similares en cuanto a intensidad parasitaria y excreción fecal de huevos. Esta falta de inhibición podría tener dos explicaciones no excluyentes. La primera es que el PEG administrado no hubiera conseguido unirse a todos los taninos condensados presentes en el brezo y, por tanto, la cantidad necesaria de éstos para provocar el efecto antihelmíntico sea muy baja. La segunda es que haya otros componentes del brezo, además de los propios taninos condensados (otros polifenoles o compuestos diferentes), que sean responsables de parte del efecto antihelmíntico. La reducción parcial de la excreción de huevos de nematodos gastrointestinales en los animales que consumen taninos condensados y a los que se administra PEG ya ha sido observada con anterioridad (Kabasa et al.,2000; Akkari et al., 2008b, Marie-Magdeleine et al., 2010), así como el incremento de la humedad en las heces de los animales a los que se les administra PEG (Akkari et al., 2008a).

Los experimentos *in vitro* demostraron que la incubación con los extractos de brezo causaron un descenso en la tasa de eclosión de huevos de los nematodos gastrointestinales estudiados. El descenso es intenso con los huevos de *T. colubriformis* (hasta 100%), intermedio con los de *T. circumcincta* (hasta 67%) y escaso con los de *H. contortus* (hasta 12 %). Se observan además diferencias entre parásitos y extractos, pero no son homogéneas. Quizás *E. cinerea* sea el extracto que muestre el efecto más constante con todos los parásitos. La incubación con todos los extractos causó un descenso significativo (hasta 100%) del desenvainamiento larvario en las tres especies de nematodos gastrointestinales investigadas. El desenvainamiento parece ser el proceso más sensible a la acción del brezo, siendo menores las dosis necesarias para producir este efecto que para inhibir la eclosión de los huevos. Se ha observado que las larvas infectantes parecen ser muy sensibles a la acción del brezo, causando éste alteraciones en el desenvainamiento incluso a concentraciones muy bajas. Estos resultados *in vitro* explican la disminución en la tasa de implantación de los nematodos estudiados en las cabras que consumen brezo, más si cabe cuando se demuestra que ese descenso se ha producido ya a los cinco días postinfección. La acción sobre el proceso de desenvainamiento y el consiguiente descenso de la tasa de implantación también explicaría (aunque pueden existir otras causas) la menor intensidad parasitaria observada en los animales suplementados con brezo en los experimentos de pastoreo. Todos los extractos indujeron también mortalidad en los vermes adultos, tanto de *T. colubriformis* como de *T. circumcincta*, aunque a dosis iguales o superiores a 600 µg/mL.

Tanto los experimentos en condiciones controladas como los experimentos *in vitro* han contribuido a explicar las observaciones realizadas en los experimentos de pastoreo. En los experimentos de campo se observó un descenso de la intensidad parasitaria en los animales suplementados que también se observó en cabras infectadas experimentalmente con *T. colubriformis* y *T. circumcincta* cuando se administró brezo durante la infección. En el caso de *T. circumcincta*, además se demostró que es durante los cinco primeros días de implantación cuando sucede el efecto. El desenvainamiento de las larvas infectantes tiene lugar durante este periodo y es un proceso clave para la implantación. Las larvas sin desenvainar son incapaces de penetrar en la mucosa del hospedador, es más, si este proceso se alarga las larvas infectantes podrían ser expulsadas con las heces (Hertzberg et al., 2002). El descenso en la eclosión de huevos y, por lo tanto, el número de futuras larvas infectantes, también explicaría la menor intensidad parasitaria de los animales suplementados en los experimentos de campo. Por último, la inducción de la mortalidad de vermes adultos observada mediante ensayos *in vitro* (AMIA), podría ser causa directa de una disminución en la intensidad parasitaria. Sin embargo, cuando se administró el brezo a cabras con una población patente de nematodos gastrointestinales, el descenso de la intensidad parasitaria fue muy discreto en *T. colubriformis* y prácticamente nulo en *T. circumcincta*. Los experimentos *in vitro* corroboraron un efecto directo sobre los vermes adultos. Sin embargo, los resultados del AMIA mostraron como solo se produce mortalidad de los vermes cuando el extracto está más concentrado. Estas concentraciones podrían no ser siempre alcanzadas en condiciones *in vivo*, lo que podría explicar el escaso descenso de la intensidad parasitaria cuando se administró brezo de forma curativa en cabras con una infección patente. Por otro lado, el descenso en la excreción de huevos podría estar causado por un descenso de la intensidad parasitaria o por un descenso en la fecundidad de los vermes. Si se tratase del primer caso, en las infecciones experimentales observaríamos un descenso de la intensidad parasitaria y no es así en todas las ocasiones. Con estos resultados, se podría sugerir que existe un efecto directo del brezo sobre los vermes adultos, induciendo cierta mortalidad, pero que además debería estar acompañada de otros efectos sobre el parásito que aunque no provoquen su muerte sí disminuyan su desarrollo y fecundidad.

En conjunto, estos experimentos han abordado la búsqueda de nuevas herramientas de control antihelmíntico no basadas en la quimioprofilaxis, sino centrándose en el uso de nutraceúticos. Se pretendió conocer los efectos del consumo de brezo (con un contenido moderado en taninos condensados) en el control de las nematodosis gastrointestinales en cabras. Se estudió ese efecto sobre el parásito de forma directa (huevos, larvas y adultos) y sobre el binomio parásito-hospedador (intensidad parasitaria, excreción de huevos, desarrollo de los parásitos, ganancia de peso de los animales). Una vez comprobado que el brezo mostraba efecto antihelmíntico en condiciones de campo, se intentó dilucidar frente a qué parásitos o estadios parasitarios era efectivo, a qué concentraciones y qué componentes (solo los taninos condensados u otros polifenoles) eran efectivos, siendo la finalidad última de este trabajo su aplicación práctica en el futuro. Aunque los experimentos realizados han aportado valiosa información acerca de cómo se puede utilizar el brezo para el control de las nematodosis gastrointestinales y también del porqué y el cómo de su actividad, hay que tratar los resultados obtenidos con precaución, siendo los autores de este trabajo conscientes de sus limitaciones. La principal limitación de los experimentos en pastoreo es que no se controlan dos importantes parámetros: la cantidad parásitos con la que se infecta cada animal y la cantidad de brezo que consume cada uno en cada momento (que se acentúa aún más al tratarse de animales muy jerárquicos). La distribución de las larvas infectantes en el pasto no es uniforme, por lo tanto las cabras se infectan de forma irregular y aleatoria. Por esta razón no se controla este parámetro y esto es un problema a la hora de comparar tanto los recuentos de huevos en heces como las cargas parasitarias. Por otro lado, existe una gran variabilidad individual en cuanto a la resistencia a las nematodosis gastrointestinales, lo que provoca grandes desviaciones en la intensidad parasitaria y excreción fecal de huevos. Como resultado, aunque se observaron grandes diferencias, tanto en la intensidad parasitaria como en la excreción fecal de huevos entre los grupos estudiados, las desviaciones son elevadas y se necesita un mayor número de animales para asegurar que son significativas esas diferencias observadas y, por tanto, atribuibles al tratamiento. En los experimentos *in vivo* en condiciones controladas, sí se conocen los dos parámetros anteriores (se infecta con un número de larvas infectantes conocido y la cantidad de brezo que se administra individualmente a cada animal es en función de su peso). Sin embargo, como en cualquier experimento *in vivo* sigue existiendo una gran variabilidad individual y no se pueden separar el efecto directo (sobre el propio parásito) del indirecto (mejora de la resiliencia y/o resistencia del hospedador). Además, tanto en los experimentos controlados como en los de pastoreo se usa la excreción fecal de huevos como una medida indirecta de la intensidad parasitaria. Aunque existe una buena correlación entre los recuentos de huevos en heces y la intensidad parasitaria (muy elevada en *H. contortus*, media en *T. colubriformis* y menor en *T. circumcincta*) no siempre es exacta y tiene un componente estacional (Cringoli et al., 2008). Incluso con intensidad parasitaria similar, hay animales que presentan signos clínicos y otros que las toleran bien y no presentan ninguno. En los experimentos *in vitro* se separa por completo hospedador y parásito, por lo tanto, todos los efectos estudiados serán directos. Esto es una ventaja, pero también un inconveniente ya que es prácticamente imposible reproducir exactamente las condiciones que se dan en el hospedador. Es especialmente complicado en vermes adultos, ya que el rango de temperatura al que pueden vivir es muy estrecho y no se dispone de un medio en el que se puedan alimentar de forma similar a como lo hacen en el tracto gastrointestinal del hospedador. También hay que tener en cuenta que las concentraciones de extracto utilizadas reproducen solo de forma aproximada lo que sucede *in vivo*. Aunque existe una alta correlación entre los efectos observados *in vitro* e *in vivo*, hay que ser muy cuidadoso a la hora de extrapolar los resultados obtenidos a situaciones *in vivo* y más todavía a lo que sucede en condiciones de campo (Paolini et al., 2004). Los ensayos *in vitro* tampoco tienen en cuenta las modificaciones que pueden suceder en las plantas al pasar por el aparato digestivo ni su toxicidad. El contenido en taninos de una planta no puede ser el único criterio utilizado para su selección. Existen varios métodos para su cuantificación, estando más indicados algunos que otros dependiendo de si se pretende cuantificarlos con fines químicos, nutricionales o parasitológicos (Álvarez del Pino et al., 2005). También existen diferencias entre el tipo de taninos que contienen y su actividad. Independientemente de los resultados obtenidos, la eficacia esperada de la inclusión en la dieta de una planta, forraje o preparación que los contenga (por ejemplo pellets) estará lejos de la de un fármaco, donde se ha incluido un principio activo a una concentración elevada, con una disponibilidad alta, donde además se ha considerado su velocidad de absorción y se ha buscado la vía de administración más efectiva.

Aún teniendo en cuenta todas las precauciones anteriores, parece claro que el brezo (con un contenido moderado en taninos) en la cantidad que lo consume el ganado caprino no parece tener efectos antinutritivos y que su consumo reduce la excreción fecal de huevos de los nematodos gastrointestinales, la implantación de las larvas infectantes, la eclosión de los huevos y puede reducir la fecundidad y desarrollo de los vermes adultos e incluso inducir su muerte. Esto implica que podría contribuir de manera efectiva al control de las nematodosis gastrointestinales en el ganado caprino. Estos resultados presentan bastantes similitudes con los numerosos trabajos que se han llevado a cabo con otras plantas (incluyendo árboles, arbustos y especies herbáceas) tanto en climas tropicales como templados y todas ellas con elevado o moderado contenido en taninos condensados. La gran mayoría de estos estudios coinciden en su efecto sobre la excreción fecal de huevos, en muchos casos junto a una disminución de la intensidad parasitaria y/o la fecundidad y desarrollo de los vermes (se muestra una revisión de estos trabajos en Moreno-Gonzalo et al., 2013, Capítulo I de esta Tesis).

Con estos resultados y dada la abundancia de brezo, la alta apetencia que tiene por él el ganado caprino y su capacidad para consumir una cantidad elevada de taninos (Silanikove et al., 1996), el consumo de brezo por las cabras, acompañado por otras medidas, podría contribuir al control de las nematodosis gastrointestinales, ralentizando además la aparición de cepas de nematodos gastrointestinales resistentes y sin generar ningún tipo de residuo químico.

Se ha observado que los herbívoros no solo ajustan su dieta a sus necesidades nutricionales, sino que pueden adaptar su comportamiento alimenticio para combatir el parasitismo ya sea evitando áreas de pastoreo contaminadas, seleccionando dietas que mejoren su resistencia a los parásitos o seleccionando alimentos con propiedades antiparasitarias (*self-medication*) (Hutchings et al., 2003). Se cree que los animales que han experimentado una infección parasitaria aumentan la cantidad de taninos ingeridos en la dieta (Lisonbee et al., 2009; Villalba et al., 2010). En este sentido, también se sabe que existe una gran diversidad de productos presentes en las plantas que contienen propiedades inmunomoduladoras o terapéuticas que pueden ser aprovechadas por los herbívoros si disponen de sistemas de manejo que se lo permiten (Provenza y Villalba, 2010). Además los animales seleccionan estas plantas a pesar de contener metabolitos secundarios potencialmente tóxicos en lugar de otras más nutritivas o seguras y este comportamiento puede pasar de generación en generación (Villalba y Landau, 2012).

Como ya se ha puesto de manifiesto en el comienzo de esta discusión, más que en la erradicación de estas parasitosis el control consiste en mantenerlas en unos márgenes asumibles en términos no solo económicos, sino también de bienestar animal. La cantidad de variables que afectan a estos procesos son múltiples y abarcan al hospedador (fase parásita): tratamientos antihelmínticos, resistencia genética, estado fisiológico y nutrición; y al ambiente (fase de vida libre): tipo de heces, aireación, temperatura y humedad, tipo de suelo y vegetación. La utilización de plantas bioactivas para el control de las nematodosis gastrointestinales se encuadra dentro de los métodos no farmacológicos para el control de las nematodosis gastrointestinales, como son la selección genética de animales resistentes (Bishop, 2012), el desarrollo de vacunas, métodos biológicos como la utilización de hongos nematófagos (Eysker et al., 2006), el manejo de pastos y el manejo de la alimentación. Por último, debe destacarse que el uso de cualquier estrategia específica para el control de los nematodos gastrointestinales, sea quimioterapéutica o no, no será sostenible a largo plazo si se utiliza de forma aislada (Waller, 2006).

CAPÍTULO VI

Conclusiones/Conclusions

Sobre el estudio del efecto antihelmíntico y nutritivo del consumo de brezo en cabras en pastoreo: El consumo de brezo por cabras en pastoreo puede constituir hasta 20-30% del total de su dieta. Su ingestión no se asocia a efectos antinutritivos. Por el contrario, se observan efectos beneficiosos como el aumento de la producción de ácidos grasos volátiles en el rumen. La suplementación con brezo incrementa, además, la resiliencia de las cabras frente a la infección por nematodos gastrointestinales, registrándose una menor tasa de mortalidad y un incremento de la condición corporal y peso vivo de los animales suplementados respecto a los testigos no suplementados a lo largo de la estación de pastoreo. La suplementación con brezo reduce la excreción fecal de huevos de nematodos gastrointestinales. Esta reducción es superior cuando la intensidad parasitaria es elevada (especialmente al final de la estación de pastoreo y con alta carga ganadera).

Sobre el efecto antihelmíntico del consumo de brezo en cabras infectadas experimentalmente *por Trichostrongylus colubriformis* y *Teladorsagia circumcincta*: El consumo de brezo durante la infección con *T. colubriformis* y *T. circumcincta* se asocia a una reducción de la implantación de las larvas infectantes de ambos parásitos. En el caso de *T. circumcincta*, el descenso de la implantación se detecta ya a los cinco días postinfección. Esta reducción temprana de la implantación de las larvas infectantes sugiere que el efecto del brezo sucede durante el desenvainamiento larvario o en la primera fase de la implantación en la mucosa. El consumo de brezo en cabras con una infección patente de *T. colubriformis* o *T. circumcincta* se asocia con una reducción de la excreción fecal de huevos de ambos parásitos. En el caso de *T. circumcincta*, esta reducción está asociada a la disminución del desarrollo y fecundidad de las hembras parásitas. La administración de un inhibidor de los taninos condensados, como es el polietilenglicol, no anula el efecto antihelmíntico del brezo, hecho que probablemente sugiere la existencia de otros compuestos implicados en esta acción o que la cantidad de taninos condensados necesaria para ejercerla es muy baja.

Sobre el estudio *in vitro* del efecto antihelmíntico de extractos de brezo sobre diferentes estadios parasitarios de *Trichostrongylus colubriformis*, *Teladorsagia circumcincta* y *Haemonchus contortus*: La incubación de extractos polifenólicos de *Calluna vulgaris*, *Erica cinerea* y *Erica umbellata*, así como una mezcla de las tres especies, con huevos y larvas infectantes de *T. colubriformis*, *T. circumcincta* y *H. contortus* se asocia con una reducción de la tasa de eclosión y la tasa de desenvainamiento en los tres parásitos investigados, siendo ésta más intensa en el caso de *T. colubriformis*. La incubación con extractos purificados, incluyendo exclusivamente taninos condensados, también reduce la tasa de desenvainamiento de las larvas de *T. colubriformis*, lo que sugiere la implicación de estos compuestos fenólicos en la acción antihelmíntica del brezo. Además, la incubación con los extractos polifenólicos de las especies de brezo antes mencionadas reduce la supervivencia de *T. colubriformis* y *T. circumcincta* adultos, pero a concentraciones más elevadas que las necesarias para inhibir la eclosión de huevos y el desenvainamiento larvario. El diferente efecto antihelmíntico observado entre los tres extractos de brezo y su mezcla sobre huevos, larvas y nematodos adultos sugiere que podrían existir distintos compuestos con efecto antihelmíntico cuyas interacciones podrían potenciar o reducir dicho efecto.

Por último, puede concluirse que a la vista de los resultados obtenidos tanto en los experimentos *in vivo* como *in vitro* desarrollados, se demuestra el efecto antihelmíntico del brezo sobre las especies más importantes de nematodos gastrointestinales presentes en el ganado caprino de nuestro país, sugiriéndose que el consumo de brezo por las cabras en pastoreo debería ser contemplado como un método alternativo o complementario en el control de las nematodosis gastrointestinales de estos animales.

On the study of the anthelmintic and nutritional effect of heather consumption in grazing goats: Grazing goats can include up to 20-30% of heather in its diet when it is available. Heather consumption is not associated with anti-nutritional effects but with a beneficial effect with the increase in volatile fatty acid production in the rumen. Heather supplementation also increases the resilience of goats against gastrointestinal nematode infection, with a lower mortality rate and a better body condition score and body weight changes of supplemented animals compared with non-supplemented animals during the grazing season. Heather supplementation reduces gastrointestinal nematode egg excretion in feces. This reduction is bigger when worm burdens are high (over all at the end of the grazing season and with high stocking rate).

On the anthelmintic effect of heather consumption in goats experimentally infected with *Trichostrongylus colubriformis* and *Teladorsagia circumcincta*: Heather consumption during the infection with *T. colubriformis* and *T. circumcincta* is associated with a reduction of the establishment of the infective larvae of both parasites. In the case of *T. circumcincta*, the decrease in the establishment is already observed five days post-infection. This early reduction of infective larvae establishment suggests that the effect of heather occurs during the larval exsheathment or during the first part of the establishment in the mucosae. Heather consumption in goats with a patent infection of *T. colubriformis* or *T. circumcincta* is associated with a reduction of fecal egg excretion of both parasites. In the case of *T. circumcincta* this reduction is associated with a decrease in the development and fecundity of adult female worms. The administration of an inhibitor of condensed tannins, such as polyetilen glycol, did not reduce the anthelmintic effect of heather. This fact suggests that there probably exist other compounds implicated in this action or that the amount of tannins necessary to produce the effect is quite low.

On the *in vitro* study of the anthelmitic effect of heather extracts on different parasitic stages of *Trichostrongylus colubriformis*, *Teladorsagia circumcincta* and *Haemonchus contortus*: The incubation of polyphenolic extracts of *Calluna vulgaris*, *Erica cinerea*, *Erica umbellata* and a balanced mixture of the three species, with *T. colubriformis*, *T. circumcincta* and *H. contortus* eggs and infective larvae is associated with a reduction of the hatching rate and larval exsheathment rate in all of the three parasites, showing higher reduction *T. colubriformis*. The incubation with purified extracts, including only condensed tannins, also reduces the exsheathment rate of *T. colubrifomis* larvae, which suggests the implication of these compounds in the anthelmintic action of heather. Furthermore, the incubation with the polyphenolic extracts of heather species mentioned before reduces the survival of *T. colubriformis* and *T. circumcincta* adult worms, but at higher concentrations than those needed to inhibit egg hatching and larval exsheathment. The different anthelmintic effect observed between the three heather extracts and the mixture on eggs, larvae and adult worms suggests that there could be different compounds with anthelmintic effects and their interactions could increase or reduce this effect.

Finally, it can be concluded that considering the results obtained in both *in vivo* and *in vitro* experiments, the anthelmintic effect of heather on the most important gastrointestinal nematode species in goats present in our country is demonstrated, suggesting that heather consumption by grazing goats should be seen as an alternative or complementary control method of gastrointestinal nematodes in these animals.

Resumen/Summary

Las nematodosis gastrointestinales de los pequeños rumiantes son las enfermedades parasitarias más frecuentes e importantes de estas especies domésticas en los sistemas de explotación extensiva. El método de control más utilizado frente a estas parasitosis es la administración repetida de fármacos antihelmínticos en las épocas de mayor riesgo. Sin embargo, la aparición de cepas de nematodos resistentes a los fármacos más utilizados y la demanda de los consumidores de productos naturales y libres de cualquier residuo químico ha estimulado la búsqueda de nuevas estrategias de control. En este sentido, el consumo de plantas bioactivas puede amortiguar la acción patógena de los parásitos, mejorando la resistencia y resiliencia del hospedador frente a estas infecciones.

El objetivo general de la presente Tesis Doctoral fue estudiar el efecto antihelmíntico y nutritivo del brezo (Ericaceae) en el ganado caprino. Para ello se realizaron secuencialmente tres tipos de estudios. En primer lugar, se estudió el efecto de la suplementación con brezo en ganado caprino en pastoreo e infectado por nematodos gastrointestinales de forma natural. A continuación, se investigó el efecto antihelmíntico del consumo de brezo en cabras infectadas experimentalmente con *Trichostrongylus colubriformis y Teladorsagia circumcincta,* dos especies de nematodos gastrointestinales con gran importancia en el ganado caprino de climas templados. Por último, se investigó mediante pruebas *in vitro* el efecto antihelmíntico de extractos de diferentes especies de brezo sobre los estadios de desarrollo de las especies de nematodos mencionadas.

En los estudios con infecciones naturales se realizaron dos experimentos. En el primero se estudió la cantidad de brezo que consumía voluntariamente el ganado caprino en pastoreo cuando existía total disponibilidad y su efecto sobre algunos parámetros parasitológicos, productivos y nutricionales. En el segundo experimento se estudió el efecto de la suplementación con brezo y la carga ganadera sobre algunos parámetros parasitológicos y productivos. Se demostró que las cabras incluyeron entre 20 y 30% de brezo en su dieta, sin observarse efectos antinutritivos, pero si beneficiosos, como el aumento de la producción de ácidos grasos volátiles en el rumen. La suplementación con brezo redujo la tasa de mortalidad y también incrementó el peso vivo y la condición corporal de los animales a lo largo del periodo de pastoreo. Además, el consumo de brezo estuvo asociado con la reducción de la excreción fecal de huevos de nematodos gastrointestinales y, en algunos casos, la intensidad parasitaria y el desarrollo de los vermes en aquellos animales suplementados. La reducción fue más importante en las cabras sometidas a una mayor carga ganadera.

Los experimentos en condiciones controladas incluyeron infecciones experimentales monoespecíficas en cabras con *T. colubriformis* y *T. circumcincta*. En ambos casos, se investigó el efecto preventivo (el animal se encuentra consumiendo brezo cuando se produce la infección) y curativo (el animal presenta una infección patente cuando empieza a consumir brezo) del consumo de brezo, sobre la tasa de implantación y desarrollo parasitario y sobre la intensidad parasitaria, la excreción fecal de huevos y el desarrollo parasitario, respectivamente. Se demostró la reducción de la tasa de implantación de ambos parásitos cuando se administraba brezo. En el caso de *T. circumcincta*, ésta se produjo en la primera fase de la implantación, siendo el desarrollo posterior y la fecundidad de las hembras parásitas significativamente menor. También se demostró una disminución de la excreción fecal de huevos de ambos parásitos en los animales que consumieron brezo, acompañada de una disminución de la fecundidad y desarrollo de los vermes en el caso de *T. circumcincta*. La administración de polietilenglicol, un inhibidor de los taninos condensados, no anuló el efecto antihelmíntico del brezo en las cabras infectadas con *T. colubriformis*, sugiriendo este hecho que hay otros compuestos implicados en este efecto o que la cantidad de taninos condensados necesarios para ejercer la acción antihelmíntica puede ser muy baja.

Por último, se estudió el efecto directo de extractos polifenólicos procedentes de diferentes especies de brezo (*Calluna vulgaris*, *Erica cinerea*, *Erica umbellata* y una mezcla de las tres a partes iguales) sobre diferentes fases de desarrollo (huevo, larva y adulto) de *T. colubriformis*, *T. circumcincta* y *H. contortus*. Para ello se utilizaron tres pruebas: ensayo de eclosión de huevos (*Egg Hatching Assay*, EHA); ensayo de la inhibición del desenvainamiento larvario (*Larval Exsheathment Inhibition Assay*, LEIA) y ensayo de inhibición de la motilidad de adultos (*Adult Motility Inhibition Assay*, AMIA). La incubación con los extractos causó una reducción en la tasa de eclosión de huevos de las tres especies. También se redujo el porcentaje de larvas infectantes desenvainadas en todas las especies estudiadas, llegando en muchos casos a inhibirse totalmente el desenvainamiento. En el LEIA de *T. colubriformis* se utilizaron además los extractos purificados (solo conteniendo taninos condensados), observándose la reducción del desenvainamiento, lo que sugiere que los taninos condensados podrían estar implicados en los efectos observados. La incubación con los extractos polifenólicos también indujo mortalidad en los vermes adultos de *T. colubriformis* y *T. circumcincta*, pero solo a las mayores concentraciones de extractos utilizadas.

En conjunto, los experimentos realizados demuestran que el brezo, consumido en las cantidades en las que lo hace el ganado caprino en pastoreo, no causa efectos antinutritivos y mejora el peso vivo

y la condición corporal de los animales, reduciendo también la mortalidad. Además, reduce la eliminación fecal de huevos de nematodos gastrointestinales y la intensidad parasitaria. Los experimentos en condiciones controladas sugieren que los efectos antihelmínticos observados en condiciones de pastoreo podrían ser debidos a la reducción en la tasa de implantación de las larvas infectantes y a una disminución de la fecundidad y el desarrollo de los vermes adultos, más que a la mortalidad de éstos. Los experimentos *in vitro* demostraron que el brezo tiene un efecto directo sobre la eclosión de los huevos, el desenvainamiento larvario y la mortalidad de vermes adultos. El desenvainamiento larvario parece ser el proceso más afectado por la acción de los extractos polifenólicos de brezo. Al usar extractos puros de brezo (que incluyen solo taninos condensados) la acción sobre el desenvainamiento persiste, sugiriendo que los taninos son responsables directos de dicho efecto. Sin embargo, al administrar a las cabras un inhibidor de los taninos condesados, como el polietilenglicol, no se anulan los efectos antihelmínticos del brezo, hecho que sugiere que podría haber otros compuestos polifenólicos implicados en la acción antihelmíntica del brezo.

Gastrointestinal nematode infections of small ruminants are the most frequent and important parasitic disease in production systems with grazing animals. The repeated administration of anthelmintic drugs in the higher risk infection periods is the most used control method. However, the appearance of nematode strains resistant to the most used anthelmintic drugs and the demand of chemical free and natural products have prompted the search of new control strategies. In this sense, bioactive plant consumption can alleviate the pathogenic action of the parasites, improving the host resilience and resistance against these infections.

The general objective of the present Doctoral Thesis was to study the anthelmintic and nutritional effect of heather (Ericaceae) in goats. For this purpose, three different studies were carried out sequentially. Firstly, the effect of heather supplementation in grazing goats naturally infected with gastrointestinal nematodes was investigated. Then, the anthelmintic effect of heather consumption in goats experimentally infected with *Trichostrongylus colubriformis* and *Teladorsagia circumcincta*, two very important species in goats in temperate areas, was studied. Finally, the anthelmintic effect of different heather species extracts on the development stages of the mentioned nematode species was investigated.

In the field experiments, two trials were performed. In the first one, the amount of heather voluntary consumed by grazing goats when it is available and its effect on some parasitological, productive and nutritional parameters was studied. In the second experiment, the effect of heather supplementation and the stocking rate on some parasitological and productive parameters was investigated. The goats included between 20 and 30% of heather in its diet, without anti-nutritional effects but with beneficial ones, such as the increase of volatile fatty acid production in the rumen. Heather supplementation reduced the mortality rate and also increased the animal live weight and the body condition score during the grazing period. Moreover, heather consumption was associated with a reduction of gastrointestinal nematode egg excretion and, in some cases, a reduction of worm burden and worm development in supplemented animals. This reduction is more important in goats under higher stocking rates.

The controlled trials included monospecific experimental infections of goats with *T. colubriformis* and *T. circumcincta*. In both cases, the preventive (the animal is consuming heather during the infection) and curative (the animal present a patent infection when is infected) effect of heather consumption, on establishment rate and worm development and on worm burden, fecal egg excretion

and worm development, respectively, were investigated. There is a reduction in the establishment rate of both parasites when heather is administrated. In the case of *T. circumcincta*, this reduction occurs during the first phase of establishment, being the subsequent development and fecundity significantly lower. A reduction in the fecal egg excretion of both parasites in animals consuming heather was demonstrated, accompanied by a decrease in worm fertility in the case of *T. circumcincta*. The administration of polyethylene glycol, an inhibitor of condensed tannins, did not reduce the anthelmintic effect of heather in goats infected with *T. colubriformis*. This fact suggests that other compounds are involved in this effect or that the amount of condensed tannins required to cause the anthelmintic action could be very low.

Finally, we studied the direct effect of polyphenolic extracts from different heather species (*Calluna vulgaris*, *Erica cinerea*, *Erica umbellata* and a balanced mixture of the three species) on different development stages (eggs, larvae and adults) of *T. colubriformis*, *T. circumcincta* and *H. contortus*. For this purpose, three assays were used: Egg Hatching Assay (EHA); Larval Exsheathment Inhibition Assay (LEIA) and Adult Motility Inhibition Assay (AMIA). The incubation with the extracts caused a reduction on the egg hatching rate of the three parasite species. The incoming larvae exsheathment rate in all the studied species was also reduced, and in some cases the exsheathment was totally inhibited. For the *T. colubriformis* LEIA purified extracts (containing only condensed tannins) were used as well. A reduction in exsheathment rates was observed, which suggest that condensed tannins could be involved in the observed effects. The incubation with the phenolic extracts also induced mortality on *T. colubriformis* and *T. circumcincta* adult worms, but only at the highest concentrations tested.

Overall, the experiments showed that heather, consumed in the amounts that grazing goats do, does not cause anti-nutritional effects and improves live weight and body condition score of the animals, also reducing mortality. In addition, heather consumption reduces gastrointestinal nematode egg excretion and worm burden. The controlled trials suggest that the anthelmintic effects of heather observed in field conditions could be due to a reduction of the establishment rate of the incoming larvae and to a decrease of adult worm fecundity and development, more than to the mortality of nematodes. *In vitro* experiments showed that the heather has a direct effect on egg hatching, larval exsheathment and adult worm mortality. The larval exsheathment seems to be the most affected process by the heather polyphenolic extracts. If purified heather extracts are used (including only condensed tannins) the action on the exsheathment persists, suggesting that tannins are directly responsible for this effect. Nervertheless, when an inhibitor of condensed tannins, such as polyetilen glycol, is administrated, the anthelmintic effect of heather is not reduced, which suggest that other polyphenolic-compounds could be involved in the anthelmintic effect of heather.

Bibliografía

Ademola, I.O., Fagbemi, B.O., Idowu, S.O., 2009. Bioseparation and activity of *Khaya senegalensis* fractions against infective larvae of *Haemonchus contortus*. Vet. Parasitol. 165, 170-174.

Akkari H., Ben Salem, H., Gharbi, M., Abidi, S., Darghouth, M.A. 2008a. Feeding *Acacia cyanophylla* Lindl. foliage to Barbarine lambs with or without PEG: Effect on the excretion of gastro-intestinal nematode eggs. Anim. Feed Sci. Technol. 147, 182-192.

Akkari, H., Darghouth, M.A., Ben Salem, H., 2008b*.* Preliminary investigations of the anti-nematode activity of *Acacia cyanophylla* Lindl.: Excretion of gastrointestinal nematode eggs in lambs browsing *A. cyanophylla* with and without PEG or grazing native grass. Small Rumin. Res. 74, 78-83.

Álvarez del Pino, M.C., Hervás, G., Mantecón, A.R.; Giráldez, F.J., Frutos, P., 2005. Comparison of biological and chemical methods, and internal and external standards, for assaying tannins in Spanish shrub species. J. Sci. Food Agric. 85, 583-590.

Álvarez-Sánchez, M.A., Perez-Garcia, J., Cruz-Rojo, M.A., Rojo-Vázquez, F.A., 2006. Anthelmintic resistance in trichostrongylid nematodes of sheep farms in Northwest Spain. Parasitol. Res. 99, 78-83.

Assis, L.M., Bevilaqua, C.M.L., Morais, S.M., Vieira, L.S., Costa, C.T.C., Souza, J.A.L., 2003. Ovicidal and larvicidal activity in vitro of *Spigelia anthelmia* Linn. extracts on *Haemonchus contortus*. Vet. Parasitol. 117, 43-49.

Athanasiadou, S., Kyriazakis, I., 2004. Plant secondary metabolites: antiparasitic effects and their role in ruminant production systems. Proc. Nutr. Soc. 63, 631-639.

Athanasiadou, S., Githiori, I., Kyriazakis, I., 2007. Medicinal plants for helminth parasite control: facts and fiction. Animal 1, 1392-1400.

Balic, A., Bowles, V.M.; Meeusen, E.N.T., 2000. The immunobiology of gastrointestinal nematode infections in ruminants. Adv. Parasitol. 45, 181-241.

Ben Salem, H., Ben Salem, L., Nefzaoui, A., Ben Said, M.S., 2003. Effect of PEG and olive cake feed blocks supply on feed intake, digestion, and health of goats given kermes oak (*Quercus coccifera* L.) foliage. Anim. Feed Sci. Technol. 110, 45-59.

Besier, R.B., 2012. Refugia-based strategies for sustainable worm control: Factors affecting the acceptability to sheep and goat owners. Vet. Parasitol. 186, 2-9.

Beynon, S.A., 2012. Potential environmental consequences of administration of anthelmintics to sheep. Vet. Parasitol. 189, 113-124.

Bishop, S.C., 2012. Possibilities to breed for resistance to nematode parasite infections in small ruminants in tropical production systems. Animal, 6, 741-747.

Bravo, L., 1998. Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. Nutr. Rev. 56, 317-333.

Brunet, S., Hoste, H., 2006. Monomers of condensed tannins affect the larval exsheathment of parasitic nematodes of ruminants. J. Agric. Food Chem. 54, 7481-7487.

Brunet, S., Aufrere, J., El Babili, F., Fouraste, I., Hoste, H., 2007.The kinetics of exsheathment of infective nematode larvae is disturbed in the presence of a tannin-rich plant extract (sainfoin) both *in vitro* and *in vivo*. Parasitology 134, 1253-1262.

Brunet, S., Jackson, F., Hoste, H., 2008. Effects of sainfoin (*Onobrychis viciifolia*) extract and monomers of condensed tannins on the association of abomasal nematode larvae with fundic explants. Int. J. Parasitol. 38, 783-790.

Brunet, S., Fourquaux I., Hoste H., 2011. Ultrastructural changes in the third-stage, infective larvae of ruminant nematodes treated with sainfoin (*Onobrychis viciifolia*) extract. Parasitol. Int. 60, 419–424.

Cala, A.C., Chagas, A.C.S., Oliveira, M.C.S., Matos, A.P., Borges, L.M.F., Sousa, L.A.D., Souza, F.A., Oliveira, G.P, 2012. *In vitro* anthelmintic effect of *Melia azedarach* L. and *Triclinia claussenii* C. against sheep gastrointestinal nematodes. Exp. Parasitol. 130, 98-102.

Cárceles, C.M., Díaz, M.S., Vicente, MS., Sutra, J.F., Alvinerie, M., Escudero, E., 2001. Milk kinetics of moxidectin and doramectin in goats. Vet. Sci. 70, 227-231.

Celaya, R., Ferreira, L.M.M., Moreno-Gonzalo, J., Frutos, P., Hervás, G., Ferre, I., García, U., Ortega-Mora, L.M., Osoro, K., 2010. Effects of heather and oat supplementation on gastrointestinal nematode infections and performance of grazing Cashmere goats. Small Rumin. Res. 91, 186-192.

Chartier, C., Pors, I., Benoit, C., 1995. Efficacy of pyrantel tartrate against experimental infections with *Haemonchus-contortus*, *Teladorsagia-circumcincta* and *Trichostrongylus-colubriformis* in goat. Vet. parasitol. 59, 69-73.

Chartier, C., Pors, I., 2004. Duration of activity of topical eprinomectin against experimental infections with *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* in goats. Vet. Parasitol. 125, 415-419.

Coop, R.L., Kyriazakis, I., 2001. Influence of host nutrition on the development and consequences of nematode parasitism in ruminants. Trends Parasitol. 17, 325-330.

Cringoli, G., Rinaldi, L., Veneziano, V., Pennacchio, S., Morgoglione, M.E., Santaniello, M., Schioppi, M., Fedele, V., 2008. Gastrointestinal strongyle faecal egg count in goats: circadian rhythm and relationship with worm burden. Vet. Res. Commun. 32, 191-193.

Díez-Banos, P., Pedreira, J., Sánchez-Andrade, R., Francisco, I., Suarez, J.L., Díaz, P., Panadero, R., Arias, M., Painceira, A., Paz-Silva, A., Morrondo, P., 2008. Field evaluation for anthelmintic-resistant ovine gastrointestinal nematodes by *in vitro* and *in vivo* assays. J. Parasitol. 94, 925-928.

Dobson, R.J., Waller, P.J., Donald, A.D., 1990. Population-dynamics of *Trichostrongylus colubriformis* in sheep: the effect of infection rate on the establishment of infective larvae and parasite fecundity. Int. J. Parasitol. 20, 347-352.

Dobson, R.J., Waller, P.J., Donald, A.D., 1990. Population-dynamics of *Trichostrongylus colubriformis* in sheep: the effect of infection rate on loss of adult parasites. Int. J. Parasitol. 20, 359-363.

EUROSTAT, 2011 http://epp.eurostat.ec.europa.eu (acceso 20.02.2013).

Eysker, M., Bakker, N., Kooyman, F.N.J., Olthuis, S.Olde, Ploeger, H.W., 2006. Effect of biological control through the daily application of spores of Duddingtonia flagrans in lambs kept under an evasive grazing system in the Netherlands. Vet. Parasitol. 140, 312-320.

FAOSTAT, 2010. http://faostat.fao.org (acceso 20.02.2013).

Frutos, P., Hervás, G., Ramos, G., Giráldez, F.J., Mantecón, A.R., 2002. Condensed tannin content of several shrub species from a mountain area in northern Spain and its relationship to various indicators of nutritive value. Anim. Feed Sci. Technol. 95, 215-226.

Gasbarre, L.C., 1997. Effects of gastrointestinal nematode infection on the ruminant immune system. Vet. Parasitol., 72, 327-337.

Gasnier, N., Cabaret, J., 1996. Evidence for the existence of a sheep and a goat line of *Teladorsagia circumcincta* (Nematoda). Parasitol. Res. 82, 546-550.

Hertzberg, H., Huwyler, U., Kohler, L., Rehbein, S., Wanner, M., 2002. Kinetics of exsheathment of infective ovine and bovine strongylid larvae in vivo and *in vitro*. Parasitology 125, 65-70.

Hoste, H., Jackson, F., Athanasiadou, S., Thamsborg, S.M., Hoskin, S.O., 2006. The effects of tannin-rich plants on parasitic nematodes in ruminants. Trends Parasitol. 22, 253–261.

Hoste,H., Torres-Acosta, J.F.J.; Aguilar-Caballero, A.J., 2008. Nutrition-parasite interactions in goats: is immunoregulation involved in the control of gastrointestinal nematodes?. Parasite Immunol. 30, 79-88.

Hoste, H., Torres-Acosta, J.F.J., 2011a. Non chemical control of helminths in ruminants: Adapting solutions for changing worms in a changing world. Vet. Parasitol. 180, 144-154.

Hoste, H., Sotiraki, S., Torres-Acosta, J.F., 2011b. Control of endoparasitic nematode infections in goats. Vet. Clin. Food. Anim. 27, 163–173.

Hoyt, P.G.; French, D.D., Miller, J.E; Williams, J.C; Hackett, G.E., Kearney, M.T., Hoyt, M.J., 1992. Evaluation of Ivermectin Against Experimental Infections of *Haemonchus contortus* and *Trichostrongylus colubriformis* in Goats. Vet. Parasitol. 42, 257-263.

Hur, S.N., Molan, A.L., Cha, J.O., 2005. Effects of feeding condensed tannin-containing plants on natural coccidian infection in goats. Asian Australas. J. Anim. Sci. 18, 1262-1266.

Hutchings, M.R.; Athanasiadou, S., Kyriazakis, I., Gordon, I.J., 2003. Can animals use foraging behaviour to combat parasites? Proc. Nutr. Soc. 62, 361-370.

Jabbar, A., Iqbal, Z., Kerboeuf, D., Muhammad, G., Khan, M.N., Afaq, M., 2006. Anthelmintic resistance: the state of play revisited. Life Sci. 79, 2413-2431.

Jackson, F., and R. L. Coop. 2000. The development of anthelmintic resistance in sheep nematodes. Parasitology 120:95-107.

Jackson, F., Hoste, H., 2010. In vitro methods for the primary screening of plant products for direct activity against ruminant gastrointestinal nematodes. In: Vercoe, P.E., Makkar, H.P.S., Schlink, A.C. (Eds.), In vitro screening plant resources for extra nutritional attributes in ruminants: nuclear and related methodologies. FAO/IAEA Springer Edition, pp. 24-45.

Jackson, F., Varady, M., Bartley, D.J., 2012. Managing anthelmintic resistance in goats-Can we learn lessons from sheep? Small Ruminant Res. 103, 3-9.

Kabasa, J.D., Opuda-Asibo, J., ter Meulen, U., 2000. The effect of oral administration of polyethylene glycol on faecal helminth egg counts in pregnant goats grazed on browse containing condensed tannins. Trop. Anim. Health Prod. 32, 73-86.

Kaplan, R.M., 2004. Drug resistance in nematodes of veterinary importance: a status report. Trends Parasitol. 20, 477-481.

Kenyon, F., Greer, A.W., Coles, G.C., Cringoli, G., Papadopoulos, E., Cabaret, J., Berrag, B., Varady, M., Van Wyk, J.A., Thomas, E., Vercruysse, J., Jackson, F., 2009. The role of targeted selective treatments in the development of refugia-based approaches to the control of gastrointestinal nematodes of small ruminants. Vet. Parasitol. 164, 3-11.

Ketzis, J.K., Vercruysse, J., Stromberg, B.E., Larsen, M., Athanasiadou, S., Houdijk, J.G.M., 2006. Evaluation of efficacy expectations for novel and non-chemical helminth control strategies in ruminants. Vet. Parasitol. 139, 321-335.

Kotze, A.C., O'Grady, J., Emms, J., Toovey, A.F., Hughes, S., Jessop, P., Bennell, M., Vercoe, P.E., Revell, D.K., 2009. Exploring the anthelmintic properties of Australian native shrubs with respect to their potential role in livestock grazing systems. Parasitology 136, 1065-1080.

Le Jambre, I.F., 1984. Stocking rate effects on the worm burdens of Angora goats and Merino sheep. Aust. Vet. J. 61, 280-282.

Lisonbee, L.D., Villalba, J. J., Provenza, F.D., Hall, J.O., 2009. Tannins and self-medication: Implications for sustainable parasite control in herbivores. Behav. Processes 82, 184-189.

Manolaraki, F., Sotiraki, S., Stefanakis, A., Skampardonis, V., Volanis, M., Hoste, H., 2010. Anthelmintic activity of some Mediterranean browse plants against parasitic nematodes. Parasitology 2010, 137, 685-696.

Marie-Magdeleine, C., Mahieu, M., Philibert, L., Despois, P., Archimède, H. 2010. Effect of cassava (*Manihot esculenta*) foliage on nutrition, parasite infection and growth of lambs. Small Rumin. Res. 93, 10-18.

Markovics, A., Cohen, I., Muklada, H., Glasser, T.A., Dvash, L., Ungar, E.D., Azaizeh, H., Landau, S.Y., 2012. Consumption of *Pistacia lentiscus* foliage alleviates coccidiosis in young goats. Vet. Parasitol. 186, 165-169.

Marley, C.L, Cook, R., Barrett, J., Keatinge, R., Lampkin, N.H, McBride, S.D., 2003. The effect of dietary forage on the development and survival of helminth parasites in ovine faeces. Vet. Parasitol. 118, 93-107.

Martínez-Valladares, M., Martínez-Pérez, J.M., Robles-Pérez, D., Cordero-Pérez, C., Famularo, M.R., Fernández-Pato, N., Castanon-Ordónez, L., Rojo-Vázquez, F.A., 2013. The present status of anthelmintic resistance in gastrointestinal nematode infections of sheep in the northwest of Spain by *in vivo* and *in vitro* techniques. Vet. Parasitol. 191, 177-181.

Max, R.A., 2010. Effect of repeated wattle tannin drenches on worm burdens, faecal egg counts and egg hatchability during naturally acquired nematode infections in sheep and goats. Vet. Parasitol. 169, 138-143.

McKellar, Q.A., 1997. Ecotoxicology and residues of anthelmintic compounds. Vet. Parasitol. 72, 413-435.

McLeod, R.S., 1995. Costs of major parasites to the Australian livestock industries. Int. J. Parasitol. 25, 1363-1367.

Min, B.R., Barry, T.N., Attwood, G.T., McNabb, W.C., 2003. The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: a review. Anim. Feed Sci. Technol. 106, 3-19.

Molan, A.L. Sivakumaran, S., Spencer, P.A., Meagher, L.P., 2004. Green tea flavan-3-ols and oligomeric proanthocyanidins inhibit the motility of infective larvae of *Teladorsagia circumcincta* and *Trichostrongylus colubriformis in vitro*. Res. Vet. Sci. 77, 239-243.

Moreno-Gonzalo, J., Ferre, I., Celaya, R., Frutos, P., Ferreira, L.M.M., Hervás, G., García, U., Ortega-Mora L.M., Osoro, K., 2012. Potential use of heather to control gastrointestinal nematodes in goats. Small Rumin. Res. 103, 60–68.

Mueller-Harvey, I., Mc Allan, A.B., 1992. Tannins: their biochemistry and nutritional properties. Adv Plant Cell Biochem Biotechnol 1, 151–217.

Mueller-Harvey, I., 2006. Unravelling the conundrum of tannins in animal nutrition and health. J. Sci. Food Agric. 86, 2010-2037.

Nieuwhof, G.J.; Bishop, S. C., 2005. Costs of the major endemic diseases of sheep in Great Britain and the potential benefits of reduction in disease impact. Anim. Sci. 81, 23-29.

Osoro, K., Martínez, A., Llorente, M., Uriarte, J., 1995. Efecto del manejo del pastoreo en el nivel de excreción de huevos de nematodos por ovinos y caprino. VI Jornadas sobre Producción Animal. ITEA, 575-577.

Osoro, K., Benito-Peña, A., Frutos, P., García, U., Ortega-Mora, L.M., Celaya, R., Ferre, I., 2007a. The effect of heather supplementation on gastrointestinal nematode infections and performance in Cashmere and local Celtiberic goats on pasture. Small Rumin. Res. 67, 184-191.

Osoro, K., Mateos-Sanz, A., Frutos, P., García, U., Ortega-Mora, L.M., Ferreira, L.M.M., Celaya, R., Ferre, I., 2007b. Anthelmintic and nutritional effects of heather supplementation on Cashmere goats grazing perennial ryegrass-white clover pastures. J. Anim. Sci. 85, 861-870.

Paolini, V., Fouraste, I., Hoste, H., 2004. In vitro effects of three woody plant and sainfoin extracts on 3rd-stage larvae and adult worms of three gastrointestinal nematodes. Parasitology 129, 69-77.

Paolini, V., De La Farge, F., Prevot, F., Dorchies, Ph., Hoste, H., 2005. Effects of the repeated distribution of sainfoin hay on the resistance and the resilience of goats naturally infected with gastrointestinal nematodes. Vet. Parasitol. 127, 277-283.

Perry,B.D.; Randolph,T.F., 1999. Improving the assessment of the economic impact of parasitic diseases and of their control in production animals. Vet. Parasitol. 84, 145-168.

Perry, B.D., Randolph, T.F., McDermott, J.J., Sones, K.R., Thornton, P.K., 2002. Investing in Animal Health Research to Alleviate Poverty. International Livestock Research Institute (ILRI), Nairobi, Kenya, p. 148.

Pomroy, W.E., Adlington, B.A., 2006. Efficacy of short term feeding of sulla (*Hedysarum coronarium*) to young goats against a mixed burden of gastrointestinal nematodes. Vet. Parasitol. 136, 363-366.

Provenza, F.D., Villalba, J. J., 2010. The role of natural plant products in modulating the immune system: An adaptable approach for combating disease in grazing animals. Small Ruminant Res. 89, 131-139.

Ramírez-Restrepo, C.A., Barry, T.N., 2005. Alternative temperate forages containing secondary compounds for improving sustainable productivity in grazing ruminants. Anim. Feed Sci. Technol. 120, 179-201.

Requejo-Fernández, J.A., Martínez, A., Meana, A., Rojo-Vázquez, F.A., Osoro, K., Ortega-Mora, L.M., 1997. Anthelmintic resistance in nematode parasites from goats in Spain. Vet. Parasitol. 73, 83-88.

Rinaldi, L.; Cringoli, G., 2012. Parasitological and pathophysiological methods for selective application of anthelmintic treatments in goats. Small Ruminant Res. 103, 18-22.

Saratsis, A., Regos, I., Tzanidakis, N., Voutzourakis, N., Stefanakis, A., Treuter, D., Joachim, A., Sotiraki, S., 2012. In vivo and in vitro efficacy of sainfoin (*Onobrychis viciifolia*) against *Eimeria* spp in lambs. Vet. Parasitol. 188, 1-9.

Shaik, S.A., Terrill, T.H., Millar, J.E., Kouakou, B., Kankan, G., Kaplan, R.M., Burke, J.M., Mosjidis, J.A., 2006. Sericea lespedeza hay as a natural deworming agent against gastrointestinal nematode infections in goats. Vet. Parasitol. 139, 150-157.

Silanikove, N., Gilboa, N., Perevolotsky, A., Nitsan, Z., 1996. Goats fed tannin-containing leaves do not exhibit toxic syndromes. Small Ruminant Res. 21, 195-201.

Silanikove, N., Gilboa, N., Nitsan, Z., 1997. Interactions among tannins, supplementation and polyethylene glycol in goats given oak leaves: Effects on digestion and food intake. Anim. Sci. 64, 479-483.

Stear, M.J., Bishop, S.C., 1999. The curvilinear relationship between worm length and fecundity of Teladorsagia circumcincta. Int. J. Parasitol. 29, 777-780.

Stear, M..J., Doligalska, M., Donskow-Schmelter, K., 2007a. Alternatives to anthelmintics for the control of nematodes in livestock. Parasitology 134, 139-151.

Stear,M. J., Fitton, L.; Innocent,G. T.; Murphy, L., Rennie, K., Matthews, L., 2007b. The dynamic influence of genetic variation on the susceptibility of sheep to gastrointestinal nematode infection. J. R. Soc. Interface 4, 767-776.

Sykes, A. R., 1994. Parasitism and production in farm-animals. Anim. Prod. 59, 155-172.

Terrill, T.H., Mosjidis, J.A., Moore, D.A., Shaik, S.A., Millar, J.E., Burke, J.M., Muir, J.P., Wolfe, R., 2007. Effect of pelleting on efficacy of sericea lespedeza hay as a natural dewormer in goats. Vet. Parasitol. 146, 117-122.

Terrill, T.H., Dykes, G.S., Shaik, S.A., Miller, J.E., Kouakou, B., Kannan, G., Burke, J.M., Mosjidis, J.A., 2009. Efficacy of sericea lespedeza hay as a natural dewormer in goats: Dose titration study. Vet. Parasitol. 163, 52-56.

Thamsborg, S.M., Jorgensen, R..J., Waller, P.J., Nansen, P., 1996. The influence of stocking rate on gastrointestinal nematode infections of sheep over a 2-year grazing period. Vet. Parasitol. 67, 207-224.

Valcárcel, F., Romero, C.G., 1999. Prevalence and seasonal pattern of caprine trichostrongyles in a dry area of central Spain. J. Vet. Med. Ser. B-Infect. Dis. Vet. Public Health 46, 673-681.

Varady, M., Papadopoulos, E., Dolinska, M., Koenigova, A., 2011. Anthelmintic resistance in parasites of small ruminants: sheep versus goats. Helminthologia 48, 137-144.

Villalba, J.J., Provenza, F.D., Hall, J.O., Lisonbee, L.D., 2010. Selection of tannins by sheep in response to gastrointestinal nematode infection.J. Anim. Sci. 88, 2189-2198.

Villalba, J.J., Landau, S. Y., 2012. Host behavior, environment and ability to self-medicate. Small Ruminant Res., 103, 50-59.

Waller, P.J., Thamsborg, S.M., 2004. Nematode control in “green” ruminant production systems. Trends Parasitol. 20, 493-497.

Waller, P.J., 2006. Sustainable nematode parasite control strategies for ruminant livestock by grazing management and biological control. Anim. Feed Sci. Technol. 126, 277–289.

Zajac, A. M., 2006. Gastrointestinal nematodes of small ruminants: Life cycle, anthelmintics, and diagnosis. Vet .Clin. North. Am. Food Anim. Pract. 22, 529-541.