



## Research paper

# Recovery of fenbendazole efficacy on resistant *Haemonchus contortus* by management of parasite refugia and population replacement



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## ABSTRACT

The recovery of fenbendazole efficacy against *Haemonchus contortus* was attempted in a sheep intensive production system, using a strategy of population replacement in which the initial absolute efficacy of fenbendazole was 0%. The strategy was based on managing the parasite populations in refugia. Firstly, the resistant parasite population was reduced by means of anthelmintic treatments with efficacious drugs (Phase I), then a new, susceptible population was introduced in summer by way of artificially infected lambs at weaning, which were left to graze on the experimental pasture for eleven months (Phase II). Lastly, the impact of the replacement strategy, in terms of benzimidazole efficacy, was measured (Phase III). Faecal egg counts from permanent lambs and worm burdens as a measure of pasture infectivity from tracer lambs were determined throughout the study. During Phase I, faecal egg counts diminished from a peak of 2968 (300–7740) epg to 0 epg at the end, while adult worm burdens of *H. contortus* were reduced from 2625 (800–5100) to 0, which showed that the treatment strategy used in Phase I was effective in reducing the resistant population. These parameters also showed that good levels of pasture contamination and infectivity were achieved in Phase II, as faecal egg counts of up to 7275 (3240–13080) epg and adult worm burdens of 500 (200–800) *H. contortus* were reached. The absolute benzimidazole efficacy on *H. contortus* estimated at 16 months post-population replacement (Phase III) was 97.58%. The results lead to the conclusion that the recovery of anthelmintic efficacy of fenbendazole against a resistant population of *H. contortus* may be achieved by means of a strategy based on management of refugia and a subsequent introduction of a susceptible population. This strategy might be translatable to other resistant nematode genera.

## 1. Introduction

The continuous advance of anthelmintic resistance is a severe limitation for sheep production (Besier and Love, 2003) and therefore currently there is a worldwide effort to search for and adopt alternative parasite control measures. The management of the parasite population in refugia is one of such alternatives. The exploitation of a resistant population in refugia and the introduction of susceptible parasites when said population is at its lowest would lead to the building up of a new, susceptible parasite population (Van Wyk and Schalkwyk, 1990). This management practice could become an on-farm alternative to recover

anthelmintic efficacy in flocks with high levels of resistance (Muchiut et al., 2018).

Lambing in Argentina occurs on pasture, typically in late-winter. The peri-parturient rise of nematode egg excretion by sheep leads to an increase in pasture infectivity that becomes the source of infection to the lambs. This peri-parturient rise is a key epidemiological factor contributing to the development of the disease (Armour, 1980), and particularly so in the case of *Haemonchus contortus*, given that the environmental conditions suitable for egg hatching (i.e. environmental temperatures > 9 °C) are present from mid-spring onwards (O'Connor et al., 2006). After weaning, lambs are fully susceptible to the infective

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larvae (L3) on pasture, and *H. contortus* is able to express its high biotic potential. This can lead to increased pasture contamination and infectivity that may quickly cause clinical disease, including the typical presentation of autumn mortalities (Sánchez and Romero, 2005).

The most adverse environmental conditions to halt *Haemonchus* larval development occurring in this region are very high temperatures and scarce rainfall, as described by Balbi (1993), Pereira et al. (2006) and Niño Uribe et al. (2018). These authors also show that larval survival is greatly dependant on the time of the year when pasture contamination occurs, with records of up to seven months of survival for contamination taking place in autumn. Pereira et al. (2006) argue that summer, being the season when both factors (i.e. high temperatures and scarce rainfall) occur simultaneously, is very efficient in reducing pasture infectivity, given that larval desiccation cause high larval mortality rates.

The purpose of this investigation was to explore and validate a biological model that takes into consideration the time when the parasite population in refugia is at its most vulnerable to the environmental conditions, in order to introduce a new, susceptible population of *H. contortus* by way of artificially-infected, recently-weaned lambs. This would allow the building up of a susceptible parasite population in autumn, thus aiding in the recovery of the efficacy of the problem anthelmintic.

## 2. Materials and methods

### 2.1. Experimental site and experimental animals

The work was carried out at the Sheep Section of the Balcarce Experimental Station, National Institute of Agricultural Technology (EEA Balcarce-INTA), in the southeast of Buenos Aires Province, Argentina. The experimental site was a 3 ha paddock managed under controlled conditions, with a pasture composed mainly of *Agropyron* spp. and an initial stocking rate of 13.3 sheep/ha. The site had a documented background of benzimidazole resistance (Entrocasso et al., 2008). Nevertheless, tests were carried out as described below to determine the level of fenbendazole resistance at the beginning of the study.

Corriedale x Texel 5 month-old lambs, initially weighing 25–30 kg, were used for each experimental phase described in section 2.3. The use of these animals and the respective procedures were approved by the Comité Institucional para el Cuidado y Uso de Animales de Experimentación (Approval Nr. 069/2016).

### 2.2. Susceptible *H. contortus* strain

A susceptible *H. contortus* strain known as the ‘CEDIVE strain’ has been shown to be 100% susceptible to fenbendazole, levamisole and ivermectin through faecal egg count reductions tests (FECRT) in two analyses (Romero, J., personal communication), and has been genotypically and phenotypically characterised as fully susceptible to benzimidazoles by Guzmán (2014). This strain has been cryopreserved in the Parasitology Laboratory, Faculty of Veterinary Sciences, National University of Central Buenos Aires Province following the methodology described by Guzmán et al. (2012). Three parasite-free lambs (‘donor’ lambs), were orally infected with 7000 L3 of this strain. Bulk faecal cultures with faeces from these lambs were set up daily for five days, commencing three weeks post-inoculation, in order to obtain L3 for the inocula needed later on in the study. Ultimately, sufficient numbers of L3 for each inoculum were obtained from the freshest faecal culture set-up.

### 2.3. Experimental design

A prospective longitudinal study was conducted, comprised of three phases.

#### 2.3.1. Phase I: Reduction of resistant parasite population in refugia (February - December, Year 1)

Forty recently-weaned lambs were used, which were naturally-infected with gastrointestinal nematode burdens. These lambs had acquired the parasite infection from grazing on the experimental site and adjacent paddocks previous to the start of the study. Twenty-eight of the lambs were used as “permanent” grazers; they were treated with closantel (Adevet C°, Vetanco, dose rate 10 mg/kg bodyweight) and re-introduced to the experimental paddock on February 20. The remaining twelve lambs were used for the initial controlled efficacy test (CET), using fenbendazole (Axilur Suspensión 10% oral°, MSD, dose rate 7.5 mg/kg bodyweight). The CET was conducted at the same time than the “permanent” grazers were introduced to the experimental paddock.

Faecal samples were taken fortnightly to determine faecal egg counts and cultured for larval identification. Every three months two lambs from the “permanent” grazers group were treated with levamisole (Fosfamisol M.V.°, Biogénesis Bagó, dose rate 8 mg/kg bodyweight), and re-introduced to the experimental paddock as “tracers”; these animals were slaughtered for determination of worm burdens in the abomasum and small intestine.

Strategic anthelmintic treatments were administered throughout this phase, using non-benzimidazole drugs such as closantel (Adevet C°, Vetanco), monepantel (Zolvix°, Novartis) or derquantel + abamectin (Startect°, Zoetis) at their respective recommended dose rates. Rotation of all these different drug classes was deemed necessary because of the high selection pressure applied by the numerous treatments at this stage. These treatments aimed to reduce the parasite population in refugia; during the first semester they were administered based on the faecal egg counts of the lambs, while monthly treatments were administered during the second semester. The remaining lambs were removed from the paddock on December 15 and the pasture was mechanically mown to aid in the elimination of any remaining L3 by allowing a deeper penetration of UV rays on the soil surface and grass desiccation.

#### 2.3.2. Phase II: Building up of susceptible parasite population (January - December, Year 2)

On January 20, forty-two parasite-free lambs were each infected with 3000 susceptible *H. contortus* L3 and introduced to the experimental paddock. The role of these lambs was to disseminate the susceptible parasite population. Faecal sampling and slaughtering of tracer lambs were conducted as in Phase I. Additionally, the FAMACHA® method was applied fortnightly to check for anaemia due to haemonchosis. Some selective anthelmintic treatments were needed throughout this phase, depending on faecal egg counts and FAMACHA score > 3. Levamisole (Fosfamisol M.V.°, Biogénesis Bagó, dose rate 8 mg/kg bodyweight) was used for these individual cases due to its low persistence, allowing the animals to be quickly exposed again to L3 on pasture. Those individual animals receiving treatment were excluded from the following samplings so that the group average numbers used to estimate the pasture contamination curve was not biased. On December 15 all remaining lambs were removed from the pasture.

#### 2.3.3. Phase III: Evaluation of population replacement strategy (January - May, Year 3)

Fifty-two parasite-free lambs were introduced to the experimental paddock on January 14, with the purpose of their becoming naturally infected with the parasite population that originated from Phase II. Faecal egg counts and FAMACHA® scores were determined as previously described. Selective treatments were administered when needed, as in Phase II. Faecal egg counts from early May confirmed the establishment of adult parasites in the animals. Consequently, twelve animals were selected to conduct the CET using fenbendazole (dose rate, 7.5 mg/kg bodyweight) on May 9.

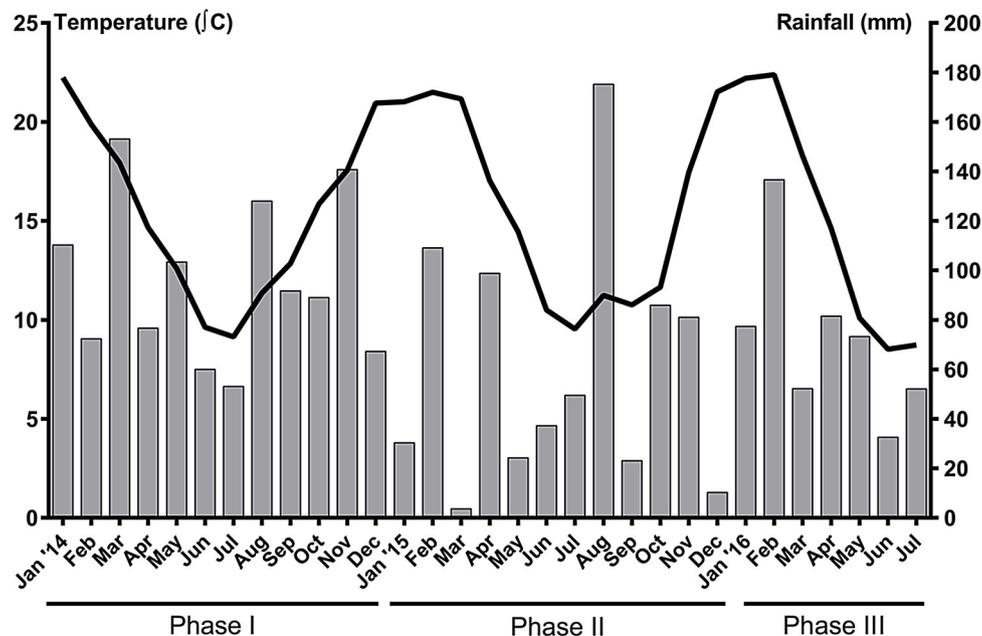


Fig. 1. Monthly average temperatures and cumulative monthly rainfall during the study. Black continuous line indicates temperature. Grey columns indicates rainfall.

Source: Agrometeorological Station, EEA-INTA Balcarce.

#### 2.4. Parasitological procedures

The following techniques and procedures were used throughout the study: Faecal egg counts using a modified McMaster technique (Roberts and O'Sullivan, 1949); faecal cultures (Henriksen and Korsholm, 1983) and identification of L3 to genus level (Niec, 1968); determination of FAMACHA® scores (Van Wyk and Bath, 2002); and CET (Vercruyse et al., 2001; Wood et al., 1995).

#### 2.5. Determination of anthelmintic efficacy

The anthelmintic absolute efficacy of fenbendazole was determined by CET, using the geometric means of the treated vs control group as described by Wood et al. (1995) and (Vercruyse et al., 2001).

### 3. Results

Fig. 1 shows the average monthly temperatures and monthly rainfall throughout the experiment.

Table 1

Absolute efficacy of fenbendazole on the initial parasite population (Phase I) and 16 months post-population replacement (Phase III) as determined by the controlled efficacy test.

Genera	Phase I			Phase III		
	Control group n = 5	Treated group n = 5	Efficacy (%)	Control group n = 6	Treated group n = 6	Efficacy (%)
<i>Haemonchus</i>	2447 <sup>a</sup> (800-5100) <sup>b</sup>	2930 (400-11500)	0	3050 (1000-7800) <sup>c</sup>	74 (0-200)	97.58
<i>Teladorsagia</i>	3380 (600-7600)	0	100	2054 (1600-3500)	0	100
<i>T. axei</i>	1452 (450-4500)	0	100	10 (0-100)	0	100
<i>T. colubriformis</i>	5137 (1950-10900)	3810 (3000-5400)	25.84	21153 (9700-56800)	22287 (12600-43900)	0
<i>Cooperia</i> <sup>c</sup>	408 (100-750)	0	100	2 (0-100)	0	100
<i>Nematodirus</i>	25 (0-500)	8 (0-300)	68.71	510 (0-25800)	368 (0-37900)	27.88

*T. axei*: *Trichostrongylus axei*; *T. colubriformis*: *Trichostrongylus colubriformis*.

<sup>a</sup> Geometric mean.

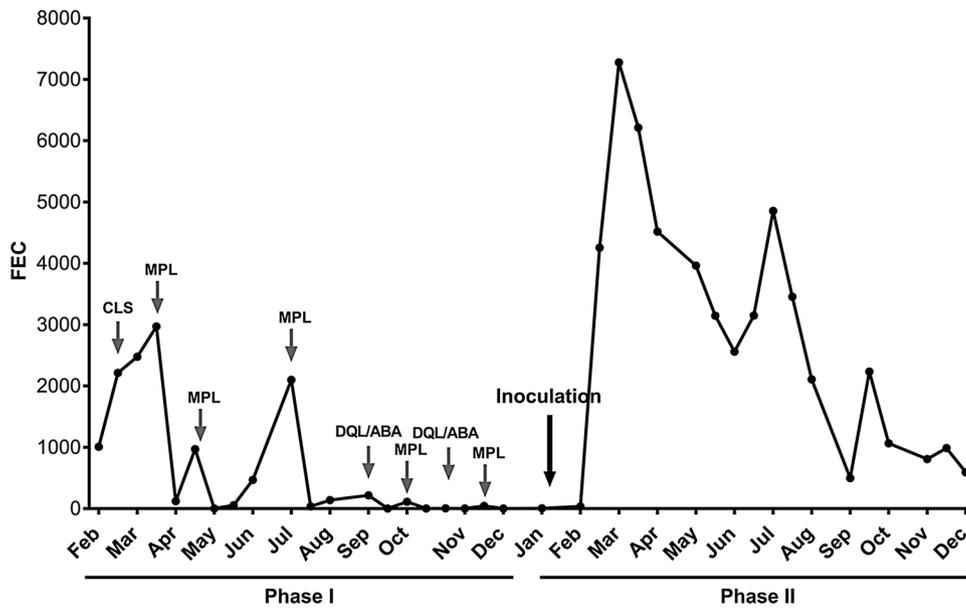
<sup>b</sup> (minimum – maximum).

<sup>c</sup> Phase I, *Cooperia curticei*; Phase III, *Cooperia* spp.

#### 3.1. Phase I

The absolute efficacy of fenbendazole at the beginning of the study is shown in Table 1. The initial CET showed high levels of resistance of *H. contortus*, *Trichostrongylus colubriformis* and *Nematodirus* spp. to fenbendazole, while *Teladorsagia circumcincta*, *T. axei* and *Cooperia curticei* were fully susceptible.

Climatic conditions were adequate for the development of free-living stages of *H. contortus*, with mean temperatures over 9 °C and over 50 mm of monthly rainfall (Fig. 1). The level of pasture contamination at the beginning of this phase was relatively high, as shown by the faecal egg counts from permanent lambs (Fig. 2), and reached a first peak of 2968 (300–7740) epg in March. The faecal egg counts increased following the reductions due to strategic treatments during the first half of the year, with two other peaks of 969 (0–3420) and 2100 (540–7600) epg recorded in late April and July, respectively. The faecal cultures corresponding to those three peaks showed that *Teladorsagia*, *Cooperia* and *Haemonchus* were the main genera present in March and July, while *Trichostrongylus* and *Haemonchus* predominated in April (Fig. 3). However, with the application of suppressive treatments during the second half of the season it was possible to lower the faecal egg counts to zero (Fig. 2).



**Fig. 2.** Dynamics of faecal egg counts in ‘permanent’ grazing lambs during Phase I and Phase II of the study. Gray arrows indicate the time of strategic anthelmintic treatment aimed to reduce the resistant parasite population in refugia during Phase I. Black arrow: Time of experimental infection of lambs with susceptible *H. contortus* L3 at the beginning of Phase II. CLS: Closantel; MPL: Monepantel; DQL/ABA: Derquantel + Abamectin.

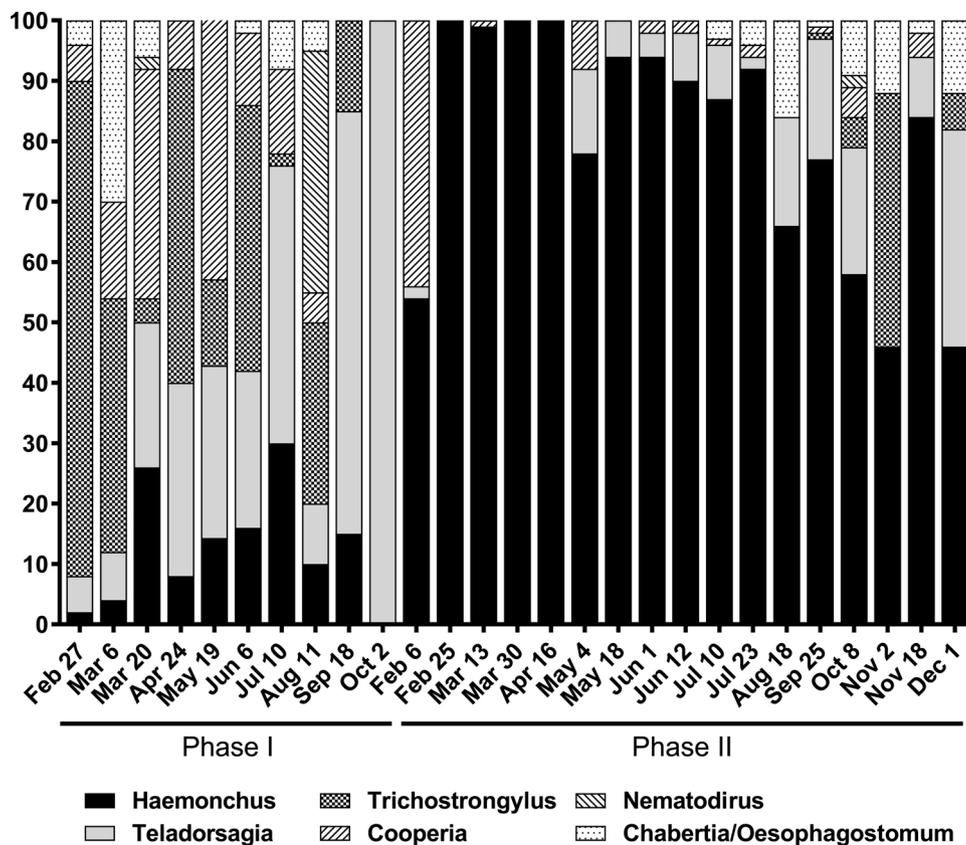
Analyses from tracer lambs grazing for three months showed that adult worm burdens of resistant *H. contortus* decreased gradually from 2625 (800–5100) worms at the beginning of the trial to 0 worms in at the end of Phase I in December (Fig. 4)

### 3.2. Phase II

During this phase the rainfall regime was more dispersed than the previous year, although humidity levels were anticipated to be sufficient for the survival and translation of L3 onto the grass. Faecal egg

counts peaked to over 7000 (3240–13080) epg at 52 days post-artificial infection and remained above 2000 epg throughout most of the year, then commenced to decrease in spring and early summer (Fig. 2). Targeted selective treatments had to be administered to seven lambs during winter, four lambs in spring and two lambs in early summer.

Adult worm burdens of *H. contortus* from tracer lambs showed the progressive build-up of what was thought to be a susceptible parasite population in refugia (Fig. 4). Examination of the first set of tracer lambs after the experimental infection with susceptible L3 revealed that 45% of the adult worm burden was comprised by *H. contortus* (data not



**Fig. 3.** Proportional representation of all parasite genera obtained from faecal cultures during Phase I and Phase II of the study.

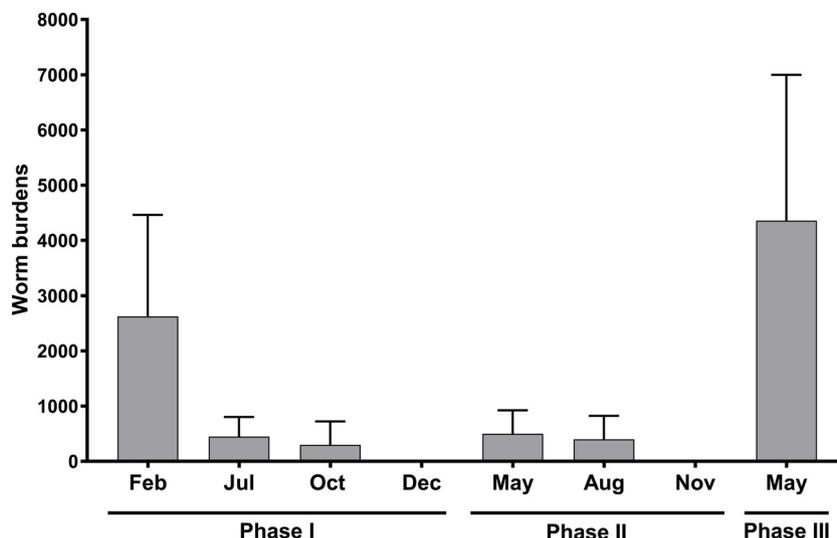


Fig. 4. Average numbers of adult *H. contortus* recovered from 'tracer' lambs during the study. These lambs grazed by pairs for three months together with the 'permanent' flock.

shown). *Haemonchus* was the genus largely predominant in faecal cultures throughout this phase (Fig. 3).

### 3.3. Phase III

As in phase I, mean temperatures and monthly rainfall in phase III were within range for the development and survival of *H. contortus* free-living stages (Fig. 1). The CET performed sixteen months after the introduction of the susceptible *H. contortus* population showed that the absolute efficacy of fenbendazole against *H. contortus* was 97.58%. *Teladorsagia circumcincta*, *T. axei* and *Cooperia* spp. were fully susceptible (100%), while *T. colubriformis* (0%) and *Nematodirus spathiger*. (27.88%) remained resistant (Table 1).

## 4. Discussion

The CET is a highly sensitive technique to discern the level of susceptibility/resistance for each parasite genus in a mixed population, and given that is still considered to be the golden standard (Coles et al., 2006), it remains the technique of choice for research studies. The initial CET showed a complete lack of efficacy of fenbendazole against *H. contortus*. The drug also failed to control *T. colubriformis* and *Nematodirus* spp. The other nematode genera found at necropsy were fully susceptible.

Given the initial levels of resistance of *H. contortus*, and taking into consideration epidemiological features of this nematode, a strategy to try to reinstate the efficacy of fenbendazole was devised. Pasture-fed sheep production in areas of the Southern Hemisphere is characterised by high levels of pasture contamination with *H. contortus* eggs at post-weaning in summer-autumn (Suarez and Busetti, 1995; Vlassoff et al., 2001). This summer-autumn period would accordingly be the ideal moment to introduce a susceptible parasite population, which could be achieved by artificially infecting recently weaned lambs. In this manner, high levels of susceptible *H. contortus* egg excretion should be ensured, which could lead to the replacement of a resistant population by a susceptible one (Muchiut et al., 2018).

Using the consideration that the L3 of *H. contortus* survive no longer than 6–7 months in temperate areas (Balbi, 1993; Niño Uribe et al., 2018; Pereira et al., 2006), a two-step strategy based on anthelmintic treatments was put into practice during Phase I, with the aim to reduce the size of refugia of resistant *H. contortus*. Treatment times in the first half of the year were predicated on results of the faecal egg counts, with the objective to lower the contamination of pasture in late summer and

autumn. The level of contamination did not decline after the first treatment with closantel. As this is a narrow-spectrum anthelmintic targeting blood-feeding parasites, it was unsurprising that other genera from the mixed parasite population such as *Teladorsagia*, *Trichostrongylus* and *Cooperia* survived the treatment, as demonstrated by the larval identification in faecal cultures. In the second half of Phase I, monthly suppressive treatments were applied in winter and spring to prevent the animals from recycling the residual resistant population in refugia. The strategy of treating animals with high efficacy anthelmintics to minimise the contamination of pasture with resistant nematodes have been used previously, either as a stand-alone strategy (Bird et al., 2001) or in combination with other procedures, such as prolonged pasture resting and pasture mowing (Aumont et al., 2002). The success of this strategy in the present study was proven by the counts of adult *H. contortus* in tracer animals, which progressively decreased to zero during this phase. The reduction of pasture infectivity was achieved even though environmental conditions were adequate for the development and survival of *H. contortus* free-living stages in this region (Balbi, 1993; Pereira et al., 2006).

One of the approaches more commonly used to reduce the *Haemonchus* parasite population in refugia before attempting to establish a susceptible one has been pasture resting for several weeks (e.g. eight or ten weeks; Van Wyk and Schalkwyk, 1990) or six months accompanied by other measures such as pasture mowing (Aumont et al., 2002; Sissay et al., 2006). Most of these studies have been carried out in tropical/subtropical areas where *H. contortus* L3 in the environment would survive no longer than two or three months (Besier et al., 2016). However, the pasture resting strategy presents a disadvantage for the animal production systems in temperate regions, where pastures would have to be closed to grazing for periods much more prolonged than in tropical/subtropical regions, because *H. contortus* L3 survive for several months (Balbi, 1993; Pereira et al., 2006). Thus, prolonged pasture resting in temperate areas might perhaps be employed in those extreme circumstances where multiple anthelmintic resistance makes it impossible to use any drug class to lower the resistant population. Other possible strategies to reduce the resistant populations in refugia are; moving animals to clean or new pastures (Miller et al., 2015), or hay-making from infected pastures that leads to high larval mortality due to grass desiccation (Moussavou-Boussougou et al., 2007).

The artificial infection of lambs with susceptible *H. contortus* L3 at the beginning of Phase II occurred in summer. The introduction of the susceptible population thus coincided with the time when the resistant parasite population in refugia was at its lowest, as suggested by Besier

and Dunsmore (1993) and Muchiut et al. (2018). The reduction of the resistant population was due to, on one hand, the anthelmintic treatment strategy applied during Phase I described above and, on the other hand, the hot and dry summer conditions that would have helped in eliminating L3 on pasture (O'Connor et al., 2006). The introduction of weaned lambs infected with susceptible *H. contortus* aimed to build up a new parasite population in refugia, as the lambs contaminated the pasture and recycled the susceptible parasites throughout Phase II. The peak of faecal egg counts following the artificial infection of the lambs in early summer was a key factor in ensuring that susceptible parasites were recycled before winter, when *H. contortus* lengthens its development (O'Connor et al., 2006). Added to all this, the few selective treatments applied during this phase helped in maintaining the susceptible *H. contortus* egg excretion above 2000 epg between mid-summer and mid-winter. The high biotic potential of *H. contortus* and its ability to cycle quickly in autumn would have resulted in a large population of susceptible parasites in refugia, from which lambs were continuously re-infected, while at the same time diluting the scarce numbers of resistant L3 in refugia that might have survived.

The introduction of a susceptible parasite population by means of experimental infections has been tried previously in other geographical areas (Aumont et al., 2002; Bird et al., 2001; Miller et al., 2015; Moussavou-Boussougou et al., 2007; Van Wyk and Schalkwyk, 1990). The appropriate time to introduce the susceptible population to pasture would definitely depend on the environmental conditions of a given location. In the case of temperate areas, the ideal time seems to be summer because of the advantages already mentioned regarding the environmental effect on the free-living stages, and also because of the usefulness of the autumn rains for the larval migration to the herbage. However, Van Wyk and Schalkwyk (1990) were unsuccessful in introducing a susceptible population in summer, although they did succeed in spring. These researchers argued that the summer failure could perhaps have been explained by the animals undergoing self-cure while harbouring susceptible worm burdens, and then subsequently getting infected with resistant L3 present on pasture. This would further reinforce the concept that the depletion of the resistant parasite population in refugia before introducing a new, susceptible one is crucial in any population replacement attempt.

During Phase II, only thirteen individual anthelmintic treatments were necessary and mortality due to haemonchosis was avoided, despite the elevated faecal egg counts, which were composed mostly of *H. contortus*, as revealed by the faecal cultures.

The CET carried out in Phase III demonstrated the success of the attempted *H. contortus* population replacement sixteen months after the susceptible population was introduced. The results indicate that it is possible to recover the efficacy to fenbendazole by applying a biological model after just one artificial infection. The absolute efficacy of fenbendazole against *H. contortus* (97.58%) shows that the original resistant population was highly diluted. On the other hand, *T. colubriformis* and *Nematodirus* spp. remained resistant to fenbendazole, indicating that the population in refugia of these two genera did not change during Phase II, when the susceptible *H. contortus* population was developing in the field and FAMACHA® was used to determine the need of selective treatments. The high worm burdens of *T. colubriformis* found at the final CET, together with the clinical manifestations observed in the animals at that time (dark diarrhoea and loss of body condition), leads to the conclusion that this parasite species can become problematic under grazing conditions when sharing its niche with *H. contortus* (Hoste and Chartier, 1993; Suarez et al., 2017).

The possibility of recovering the efficacy of anthelmintics by the application of a biological model based on parasite epidemiology and the replacement of parasite populations is an encouraging prospect, mainly due to its almost immediate applicability to livestock production enterprises. It is, however, vital that the management of such tool is accompanied by constant professional advice. Even though further and larger studies on the subject must be conducted, the results from

the present study are highly encouraging, especially because the reversion to anthelmintic efficacy was achieved with just one experimental infection. Given the current situation of anthelmintic resistance in sheep grazing systems worldwide, where the lack of drug efficacy is such a serious limitation and the associated production losses threaten the viability of such enterprises, the significance of this type of study is very high.

## 5. Conclusions

The results of the present study reinforce the idea that, by managing the parasite population in refugia, it is possible to recover the lost anthelmintic efficacy in flocks with high levels of resistance. At the same time, the results show that the *H. contortus* population replacement model is useful in temperate areas. Given the importance of other resistant parasitic species in the production performance of a flock or herd, this study additionally opens up the possibility of applying this approach to the whole, multi-species, parasite population. Hence, the population replacement model described in this study could be applied to other parasite genera/species characterised by an external life cycle and a well-defined epidemiology.

## Declaration of competing interest

The authors declare no conflict of interest.

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