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Research paper

An attempt to replace an ivermectin-resistant *Cooperia* spp. population by a susceptible one on grazing pastures based on epidemiological principles and refugia management

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ABSTRACT

The maintenance of anthelmintic-susceptible parasite refugia to delay the onset of anthelmintic resistance is an almost impossible effort in many grazing livestock production countries given that current refugia consist of already resistant parasites. Rather, efforts could be focused on replacing the resistant parasite refugia by susceptible parasite ones and implementing sustainable parasite control measures from then on. To this purpose, a trial was conducted to attempt to establish a new population of ivermectin-susceptible *Cooperia* sp. on a beef cattle farm with proven problems of ivermectin-resistant *Cooperia*. During two consecutive years, 82 (Year 1) and 100 (Year 2) recently weaned and parasite-free heifers were inoculated with 40,000 or 30,000 susceptible *Cooperia* L3, respectively, at a time when levels of resistant parasite refugia were normally low. The animals were subsequently allowed to graze on the problem pastures during autumn until the end of spring. Levels of parasitism in the animals and on pasture were monitored monthly and animals were treated with levamisole when needed. The combination of parasitological monitoring and local epidemiological knowledge was essential to determine when treatments were to be administered. No clinical signs of gastrointestinal parasitosis in the herd were observed throughout the study and unnecessary treatments were avoided. Faecal egg counts reduction tests (FECRT) and controlled efficacy tests (CET) employing worm counts were carried out at different times throughout the study to determine the clinical efficacy (FECRT) and the absolute efficacy (CET) of ivermectin, respectively. The clinical efficacy of ivermectin increased from an initial 73% to 99.4%, while the absolute efficacy increased from 54.1% to 87.5% after just two animal production cycles. The switch from a resistant parasite population to a susceptible one requires knowledge of parasitological epidemiology, especially in relation to seasonal variations of parasite populations in both the host and in refugia.

1. Introduction

Anthelmintic resistance (AR) of cattle nematodes has been described in almost all areas around the globe where grazing takes place (Sutherland and Leathwick, 2011). The first cases reported in Argentina involving avermectins date from 2000 (Anziani et al., 2001) and the most recent work published indicates that 93% of cattle farms have resistance to ivermectin and 28% have resistance to ricobendazole (Cristel et al., 2017). Anthelmintic resistance is a problem for all sectors involved in cattle production; farmers see their practical and easy way of parasite control fading away, the pharmaceutical industry finds that the length of product life-cycles is reduced, and veterinary practitioners lose an essential tool to use when providing advice on effective control programs. As a consequence, AR generates both disconcert and denial

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in all sectors, hampering any efforts and attempts to not only accept the reality of it but also to try and prevent it.

Misuse of drugs is often a main contributing factor in the development of AR. Over-simplification of and/or generalisations within control programs can lead to indiscriminate or over-frequent drug use and this can be exacerbated by the introduction of resistant populations via parasitised animals transferred from other farms (Steffan et al., 2012). Once AR is established on a given farm even abandoning the use of the implicated drug for considerable periods of time provides little meaningful reversion in efficacy (Fiel et al., 2016), mainly due to the hereditary character of AR (Wolstenholme et al., 2004), enabling the resistant nematodes to continue to cycle and be maintained on farm year after year despite the absence of chemical exposure.

Most of the published work on the management of resistance in ruminants is focused on preventing its establishment (Knox et al., 2012a; Leathwick and Besier, 2014). However, AR is a worldwide problem (Kaplan, 2004) and grazing livestock production often takes place in environments with high or concerning levels of resistance already established (Sutherland and Leathwick, 2011). It is recognised that refugia size and management can play a key role in the rate of development of AR. Preserving enough susceptible parasites in refugia is described as critical in delaying the onset of resistance (Besier, 2012). However, very little work aimed at recovering the susceptible refugia has been conducted, with all studies focusing on small ruminant production, mainly sheep. Van Wyk and Schalkwyk (1990) introduced benzimidazole-susceptible *Haemonchus contortus* to five different pastures in different seasons and showed that reversion to susceptibility occurred on pastures where parasite populations had been replaced in autumn (one pasture) and spring (two pastures). Efficacy for albendazole ranging from 96 to 99.6% was achieved in these 3 pastures, whilst the efficacy observed for the other two pastures remained low at 47–53%. Bird et al. (2001) attempted to replace a mixed population of nematodes resistant to levamisole and benzimidazoles by grazing two pastures with sheep harbouring susceptible *H. contortus*, *Teladorsagia circumcincta* and *Trichostrongylus* spp. With the exception of suspected levamisole-resistant *Trichostrongylus* spp. on one pasture, they achieved efficacy ranging from 99 to 100% for levamisole and albendazole, although they only measured clinical efficacy through faecal egg count reduction tests (FECRT). Populations of benzimidazole-resistant *T. circumcincta* were replaced by a susceptible population in France by Moussavou-Boussougou et al., (2007). They obtained post-treatment reductions in parasite burdens after the population replacement of 97–99% and used molecular technology (PCR) to confirm the results by showing that the proportion of resistant homozygotes ranged from 0 to 3%. In Argentina it was shown that after 16 months following the introduction of a benzimidazole-susceptible isolate of *H. contortus* to a pasture where the resistant population had been previously depleted, the absolute efficacy of fenbendazole (measured by controlled-efficacy tests (CET) against this nematode) improved from 0% to 98% (Muchiut et al., 2016).

In the Humid Pampa region of Argentina calves are weaned and treated with anthelmintics at the end of summer, i.e. March. They are then placed on permanent pastures that normally have undergone a resting, no-grazing period over the summer. Therefore, weaned cattle become exposed to the residual larvae on grass from the previous year that had survived the summer season (refugia), thus ensuring the reinfection of animals and, in turn, the contamination of pasture with nematode eggs throughout autumn and winter (Steffan et al., 2012). Under such management conditions, animals exposed to resistant refugia in early autumn will be cycling resistant parasites in high proportions subsequently. Local epidemiological studies have established that > 50% of the total parasite population in a whole production year originates from the first two months of grazing after weaning in early autumn. (Fiel et al., 2012; Steffan and Fiel, 1986).

However, the presence of reduced parasite refugia at weaning opens up the possibility to replace in the pasture nematode populations resistant to anthelmintics by susceptible ones. This would be achieved if calves that start grazing already harbour sufficient numbers of susceptible worms, these worms would contribute quickly to contaminate the pasture and would compete effectively with the residual resistant population, thus creating a new, more susceptible refugia. In practice, this principle would be applicable to all resistant nematode genera and all classes of anthelmintics. The present work aimed to test the above principle in the case of ivermectin-resistant *Cooperia* spp. in cattle, monitoring the process of refugia replacement through grazing of animals infected with a proven susceptible isolate at an epidemiologically appropriate time of the year.

2. Materials and methods

2.1. Experimental farms and AR background

The trial was conducted on a commercial cattle farm in the centre of Buenos Aires Province, Argentina. Resistance to ivermectin in the farm had been first detected in June 2010 by a FECRT that revealed an efficacy of 79.6%. This was confirmed in spring of 2012, again by FECRT using two groups of 15 animals each and treating one of the groups with ivermectin 1% SC at a dose of 0.2 mg/kg, while the second group remained as non-treated, control group. This test showed a clinical efficacy of 73% (CI 53.4–80.4). *Cooperia* spp. was the only genus in coprocultures post-treatment.

The experimental pasture used for the trial was a 61 ha established three-year old mixed pasture composed of tall fescue, white clover and ryegrass. The pasture was divided into 6 similar paddocks where the experimental animals grazed during autumn until the end of spring in 2013 and 2014, following a rotational scheme that allowed for a better utilisation of the forage. Large hay bales were placed on pasture as forage supplement when grass was scarce in late autumn-winter. The pasture was closed for grazing each year from December to the following March. All grazing management throughout the trial followed the farm's own animal production strategy.

2.2. Obtaining and cycling of susceptible Cooperia spp. infective larvae and parasitological procedures

The ivermectin-susceptible *Cooperia* spp. isolate used (identified as 'La Argentina' isolate) originated in 2012 from a field sampling. A FECRT conducted at that time on a commercial farm showed an efficacy of a single ivermectin treatment of 99.8% against *Cooperia* spp., while a CET revealed an absolute efficacy of 97.5% against *C. oncophora* and *C. punctata*. The susceptible isolate was cryopreserved for four years before being cycled in donor calves for the present study.

In December 2012, three parasite-free, 3–4 month-old Holando Argentino male calves, received 40,000 L3 each via latero-medial duodenal laparoscopy. The calves were kept in cattle pens with concrete floors, were fed alfalfa pellets and had access to ad-libitum water. Daily collection of faeces began four weeks post-transplant and lasted for 35 days, daily FEC during that time varied between 105 and 155 epg. Macro faecal cultures were set up daily in 60 × 80 cm black polystyrene bags and incubated at 24 °C for 15 days. Then, L3 were recovered by macro-baermannisation. Identification of L3 determined that 98% of them were *Cooperia* spp. Individual inocula of 10,000 L3 each were kept in polystyrene 20 ml-tubes and maintained at 4–6 °C for 10–15 days until their use.

The methodology described above for obtaining ivermectin-susceptible L3 was also followed the year later, except that the 'donor' calves were orally infected with L3 that had been cycled through the 'donors' the previous year and kept at 4–6 °C.

The following laboratory procedures were carried out throughout the study: FEC, using a modified McMaster technique (Fiel et al., 2011); faecal cultures according to Henriksen and Korsholm (1983); grass sampling and isolation of L3 from grass as described by Fiel et al.

Table 1
Scheme of the experimental design.

	Year 2012	Year 2013	Year 2014	Year 2015
Experimental infections		Mar 14: 20,000 L3 Mar 26: 20,000 L3	Mar 25: 30,000 L3	
Start of grazing on experimental pasture		Autumn (Mar 26)	Autumn (Mar 25)	Autumn (Mar 18)
End of grazing		Summer (Dec 6)	Summer (Dec 10)	
FECRT	Spring (Sep 25)	Spring (Nov 5)	Winter (Jun 26)	
CET		Autumn (May 10) Spring (Nov 12)	Delayed until 2015 due to low worm burdens in late winter and spring	Winter (Jul 21)
FEC and faecal cultures		Monthly	Monthly	
Pasture larval counts		Monthly	Monthly	

(2011); identification of L3 following the keys by Niec (1968); and recovery and identification of nematodes from the gastrointestinal tract at necropsy according to Fiel et al. (2011).

The procedures described by Coles et al. (1992) were followed for FERCT and CET.

2.3. Experimental design

A scheme of the experimental design is shown in Table 1.

2.3.1. Year 1

Eighty-two Aberdeen Angus heifer calves, 7–8 month-old were treated three weeks pre-weaning with levamisole (Ripercol L® 7.5%, Fort Dodge) SC, at a dose rate of 7.5 mg/kg. The heifers were then orally infected with 20,000 L3 of ivermectin-susceptible *Cooperia* spp. on March 14, 2013 (one week after anthelmintic treatment) and re-infected with another 20,000 L3 at the time of weaning, on March 26, 2013. That same day the heifers were placed on pasture to graze the 6 paddocks on a rotational basis until early-December. The pastures were sampled monthly to determine pasture infectivity and the nematode genera present. Additionally, 20 individual faecal samples were taken directly from the rectum to carry out faecal eggs counts and faecal cultures. The faecal egg counts were used to monitor indirectly the parasite burden and to determine the need of anthelmintic treatments with levamisole to avoid production losses. The decision to treat was based on the trend of monthly FEC with a requirement to not surpass 600 epg. The faecal cultures, set up by pooling the unused material from the 20 faecal samples, were used to identify the L3 by genera.

A FECRT using 15 of the 82 heifers was conducted on November 5 (day 0) and 20 (2nd collection) to monitor the advances in the recovery of the clinical efficacy of ivermectin.

The absolute efficacy of ivermectin treatments against *Cooperia* spp. was determined by a CET. Four Holando Argentino male calves, 5 month-old and 120 kg were used as 'autumn tracers'. The calves were treated with levamisole (Ripercol L® 7.5%, Fort Dodge) SC, at a dose rate of 7.5 mg/kg, and placed on the same day on the pasture to graze alongside the heifers during late-March and April. After grazing for 45 days, two tracers were treated with ivermectin (Ivomec® 1%, Merial) SC at 0.2 mg/kg dose rate, while the other two calves remained untreated. The calves were kept on a dry-lot for 14 days post-treatment then slaughtered. Their individual gastrointestinal tracts were processed in order to collect, count and identify all nematodes The same methodology as described for 'autumn tracers' was followed using another set of calves, 'spring tracers', which grazed from late-September until November 12. The CET carried out with these 'spring tracers' enabled the assessment of the degree of recovery of ivermectin efficacy after the first year of the study.

From December to the following March the pasture was rested and no animals were allowed to graze on it.

2.3.2. Year 2

One hundred Aberdeen Angus heifer calves, 7–8 month-old were treated on March 16 with levamisole (Ripercol L® 7.5%, Fort Dodge) SC, at a dose rate of 7.5 mg/kg. On March 28, 12 days after treatment with levamisole, the calves were weaned, artificially infected with a single inoculum of 30,000 ivermectin-susceptible *Cooperia* spp. L3, and placed on the same experimental pasture used in Year 1. The animals grazed on rotational basis the 6 paddocks until early-December 2014. Monthly sampling of pasture and faeces was carried out as explained in Section 2.3.1.

A follow-up FECRT to check out the status of the clinical efficacy of ivermectin was conducted on June 26 (day 0 treatment) and July 11 (day 15 collection).

As it was not possible to carry out a CET at the end of 2014, the trial was extended to the following mid-winter, whereupon the final CET was conducted. Ten recently weaned Aberdeen Angus male calves were used as 'final tracers'. The calves were treated with levamisole (Ripercol L® 7.5%, Fort Dodge) SC at a dose rate of 7.5 mg/kg, and placed the same day on the experimental pasture. They grazed from March 18 to July 21, 2015, together with calves which were of the same production cycle but had not been part of the trial up to this point. After the grazing period, five tracers were treated with ivermectin (Ivomec® 1%, Merial) SC at 0.2 mg/kg dose rate, while the other five calves remained untreated. The procedures for holding the animals and obtaining the worm burdens were as explained in Section 2.3.1. This second CET determined the absolute final efficacy of ivermectin after two production cycles, and confirmed whether the population replacement process resulted in the establishment of a new and stable susceptible parasite population.

3. Results

3.1. Pasture contamination and infectivity

3.1.1. Year 1

The faecal egg counts of the heifers in 2013, representative of the level of pasture contamination, are shown in Fig. 1. They started low and rapidly increased during the autumn until reaching a high level of 714 epg in June, at which point treatment with levamisole was needed. The FECs subsequently dropped to undetectable or negligible levels after the treatment, climbing back up moderately to a maximum of 234 epg without needing any further anthelmintic treatment. The levels of L3 on pasture, i.e. pasture infectivity, showed a similar rising trend during the first half of the trial, decreasing later towards late-winter and early-spring. The decrease coincided with a period of scarce rainfall, with a late increase in infectivity occurring during the last two months in response to a period of abundant rainfall (Fig. 1). Regarding the parasite genera present during this period (Table 2), *Cooperia* spp., with a high predominance of *C. oncophora*, dominated in faecal cultures until late winter, reducing its presence in the spring. The dominant

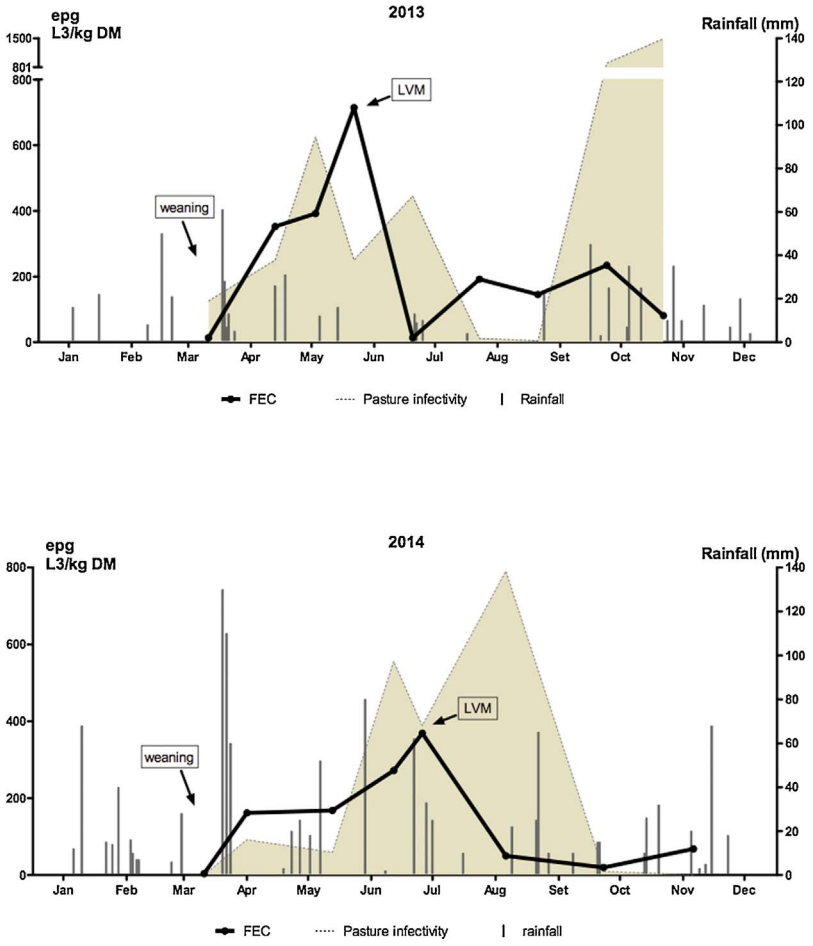

Fig. 1. Levels of FEC, larval infectivity on pasture and rainfall during Year 1 (2013) and Year 2 (2014). Rainfall data was obtained from records at the experimental site (courtesy of personnel at 'Santa Dominga' farm).

appearance of this genus on pasture lasted practically the whole year.

3.1.2. Year 2

During 2014 the faecal egg counts showed a steady increase until mid-winter, when it was necessary to treat the heifers with levamisole. After treatment the faecal egg counts remained very low (Fig. 1). The levels of pasture infectivity showed a similar trend with the highest levels also occurring in mid-winter, coinciding with abundant rainfall. Unlike the first year there was no increase in the numbers of L3 on pasture in spring despite suitable rainfall. As seen in Year 1, the presence of *Cooperia* spp. – largely dominated by *C. oncophora* – prevailed over other parasite genera (Table 3).

3.2. Monitoring of anthelmintic efficacy (resistance status)

The clinical efficacy of ivermectin was monitored by FECRT (Table 4). The initial FECRT performed on the herd in the spring of 2012, prior to this study, demonstrated a clinical efficacy of 73% (CI 53.4–80.4). The FECRT at the end of the grazing period in Year 1, i.e. November 2013, demonstrated that the clinical efficacy had increased to 94.7% (CI 80.8–98.5). This value increased further in July 2014, to 99.4% (CI 98.1–99.8). The absolute efficacy was measured throughout the study by means of CET (Figs. 2 and 3). Fig. 2 shows that throughout the trial the only nematode genus resistant to ivermectin was *Cooperia*, while all the other genera present were fully susceptible. The CET from the 'autumn tracers' (Fig. 3) that grazed with the experimental herd in early 2013 demonstrated that the initial absolute efficacy of ivermectin against *Cooperia oncophora* and *C. punctata* was 54.1%. This efficacy increased to 75.7% by the end of Year 1, as demonstrated by the CET carried out on the 'spring tracers'. The final CET was performed in mid-autumn 2015, two and half years after the experiment was initiated. The results indicated that the absolute efficacy of ivermectin against *C. oncophora* and *C. punctata* was 87.5%.

4. Discussion

The FECRT results demonstrated that the clinical efficacy of ivermectin quickly increased in Year 1 from an initial 73%–94.7% despite a 95% LCL of 80.8. The efficacy recovery consolidated in Year 2 with the final FECRT demonstrating an efficacy of 99.4% with a 95% LCL of 98.1. It is known that the sensitivity of FECRT to detect AR is restricted. Martin et al. (1989) determined that by the time clinical efficacy has signalled the possibility of resistance, the resistant population already exceeds 25%. Despite this limit the FECRT is still the most used method worldwide to diagnose and monitor for AR (Presidente, 1985; Taylor et al., 2002), especially under field conditions.

The current temporary lack of validated molecular techniques to characterise field parasite populations resistant to macrocyclic lactones (De Graef, 2013; Kotze et al., 2014) dictates that CET needs to be used in such studies to confirm the findings, adding not only a tremendous monetary cost but also animal welfare considerations. The CET results seen in this study confirmed the efficacy recovery of ivermectin, increasing from 54.1% initially to 75.6% in Year 1 and further to 87.5% at the end of the experiment. These results corroborate and confirm those seen in the FECRT providing confidence that the improvements in efficacy seen were real.

The improvement in treatment efficacy seen over the two years strongly suggests that a significant degree of population change occurred as a result of the inoculations. The inoculation of the heifers was followed by relatively rapid FEC increases during the first two months

Table 2
Proportional distribution of parasite genera in coprocultures and pasture for Year 1.

	Parasite genera (%)	
	Coprocultures	Pasture
March	***Cooperia 70*** *Ostertagia 6* *Trichostrongylus 8* *Haemonchus 16*	***Cooperia 50*** *Haemonchus 50*
April	***Cooperia 74*** *Ostertagia 6* *Trichostrongylus 2* *Haemonchus 16* *Oesophagostomum 2*	***Cooperia 70*** *Haemonchus 30*
May	***Cooperia 75*** *Ostertagia 6* *Trichostrongylus 9* *Haemonchus 7* *Oesophagostomum 3*	***Cooperia 80*** *Ostertagia 10* *Trichostrongylus 7* *Haemonchus 3*
June (AT)	***Cooperia 63*** *Ostertagia 24* *Haemonchus 6* *Oesophagostomum 7*	***Cooperia 67*** *Ostertagia 33*
July	**nd**	***Cooperia 85*** *Ostertagia 15*
August	***Cooperia 64*** *Ostertagia 14* *Trichostrongylus 10* *Haemonchus 6* *Oesophagostomum 6*	***Cooperia 64*** *Ostertagia 36*
September	***Cooperia 45*** *Ostertagia 27%* *Trichostrongylus 18%* *Haemonchus 3%* *Oesophagostomum 7%*	***Cooperia 50*** *Ostertagia 50*
October	***Cooperia 50*** *Ostertagia 44* *Trichostrongylus 2* *Oesophagostomum 4*	***Cooperia 58*** *Ostertagia 42*
November	***Cooperia 14*** *Ostertagia 10* *Trichostrongylus 28* *Haemonchus 46* *Oesophagostomum 2*	***Cooperia 41*** *Trichostrongylus 36* *Ostertagia 23*

AT: Anthelmintic treatment with levamisole.
nd: no data because of low FEC.

Table 3
Proportional distribution of parasite genera in coprocultures and pasture for Year 2.

	Parasite genera (%)	
	Coprocultures	Pasture
March	**nd**	**nd**
April	***Cooperia 80*** *Ostertagia 8* *Trichostrongylus 2* *Haemonchus 6* *Oesophagostomum 4*	***Cooperia 100***
May	***Cooperia 86*** *Ostertagia 10* *Haemonchus 4*	***Cooperia 100***
June	***Cooperia 78*** *Ostertagia 2* *Haemonchus 20*	***Cooperia 100***
July (AT)	***Cooperia 64*** *Ostertagia 16* *Haemonchus 16* *Oesophagostomum 4*	***Cooperia 72*** *Ostertagia 28*
August	***Cooperia 50*** *Ostertagia 46* *Oesophagostomum 4*	***Cooperia 56*** *Ostertagia 42* *Trichostrongylus 2*
September[1]		
October	**nd**	*Ostertagia 100*
November	***Cooperia 48*** *Ostertagia 44* *Trichostrongylus 2* *Oesophagostomum 4*	**nd**

nd: no data because of low FEC (coprocultures) or null result (pasture).
AT: Anthelmintic treatment with levamisole.
[1] No sampling due to continuous bad weather and impossibility to reach the farm.

post-inoculation in both experimental years. This increase was more pronounced in Year 1 than in Year 2 (mean epg values reached of almost 400 and 168 epg respectively). Presumably the eggs seen were mostly generated by worms from the experimental infections as the timing of the inoculations corresponded to periods of the year when the residual L3 on pasture from the previous year grazing cattle would have been quite low, due to the high mortality of free-living stages likely over the summer period. Post-weaning faecal egg counts from naturally infected pastures would normally require more than two months of continuous re-infection to create a sustained high FEC level (Fiel et al., 2013). In fact, the development time from egg to L3 in faeces in autumn takes 3–4 weeks (Fiel et al., 2012).

The coprocultures results demonstrated a high presence of *Cooperia* spp. (proportionally larger in the second year) consistent with the species inoculated. There was a similar proportion of *Haemonchus* to *Cooperia* seen on pasture in Year 1 but this turned quickly towards a large majority of *Cooperia* L3 after the inoculations. *Cooperia* spp. L3 were also the only genus detected on pasture during the first three months of Year 2.

The FECs observed were generally consistent with the pasture infectivity results (level and composition). Year 1 pasture infectivity levels increased from 100 L3/kg DM to over 600 L3/kg DM by two months post-weaning. This was reflected in the FEC, which reached a high level of 714 epg in June. However, abundant rainfall late in spring contributed to an increase in the pasture infectivity in October and November (900 and 1500 L3/kg DM respectively) which was not reflected in the FEC seen (moderate maximum of 234 epg). This lack of parasite expression (pasture to animal) was probably a function of the developing immunity seen with the increasing age of the grazing animals, combined with the good nutritional levels provided by the high quality and quantity of pasture present in spring (Steffan et al., 2012). In contrast, initial pasture infectivity levels during Year 2 remained lower than 100 L3/kg DM despite a more abundant rainfall than in the previous year. The high levels of rainfall recorded during the weeks before weaning potentially would have released L3 from the faecal pats to the surrounding grass, enabling the adverse conditions of summer to have a greater negative effect, i.e. heat and desiccation.

There were no clinical signs of gastrointestinal parasitosis in the herd throughout the experiment. The control strategy was based on parasitological monitoring (FEC, coprocultures and pasture infectivity) and epidemiological knowledge of the helminths involved. This approach was extremely useful for determining the best time for anthelmintic treatment during the period from weaning (6–7 month-old) to 14–15 months of age, enabling importantly the avoidance of unnecessary treatments (Steffan et al., 2013, 2005). This is especially relevant in production systems to delay the development of AR (Anziani and Fiel, 2015).

It is acknowledged that refugia management is essential in any program of sustainable parasite control (Knox et al., 2012b) and most published works remark on the importance of preserving the susceptible refugia as a way to delay the onset of resistance (Besier, 2012; Leathwick and Besier, 2014). In the present case in many countries it would be more appropriate, perhaps, to talk in terms of the recovery of

Table 4
Determination of clinical efficacy of ivermectin in heifers by FECRT (n: 15/group).

	Previous to Year 1 (spring 2012)		Spring Year 1		Winter Year 2	
	Control	IVM	Control	IVM	Control	IVM
	280	0	240	60	600	0
	260	60	240	0	280	0
	200	0	120	80	420	0
	420	40	100	0	1460	0
	220	40	120	0	500	20
	240	40	140	0	280	0
	540	120	140	0	1240	0
	300	140	300	0	380	0
	180	120	140	0	700	0
	220	120	440	0	720	20
	100	0	100	20	860	0
	280	200	140	0	520	0
	200	60	160	0	580	20
	240	100	100	0	640	0
	920	200	520	0	680	0
Average	**306.7**	**82.7**	**200**	**10.7**	**657.3**	**4**
FEC red. (%)		**73.0%**		**94.7%**		**99.4%**
95% UCL		*80.4*		*98.5*		*99,8*
95% LCL		*53.4*		*80.8*		*98,1*
Parasite genera (%)	*Oste 20* *Haem 8* *Trich 28* *Coop 44*	*Coop 100*	*Oste 10* *Haem 46* *Trich 28* *Coop 14* *Oeso 2*	*Coop 100*	*Oste 2* *Haem 20* *Coop 78*	–

UCL: Upper Confidence Limit. LCL: Lower Confidence Limit.
Oste: *Ostertagia*, Haem: *Haemonchus*, Trich: *Trichostrongylus*, Coop: *Cooperia*, Oeso: *Oesophagostomum*.

a susceptible refugia rather than the maintenance of it. In countries like Argentina, refugia already consists mostly of resistant parasites due to the extremely high numbers (95.5%) of cattle farms with AR (Cristel et al., 2017). The possibility of recovering susceptible refugia through establishing a new susceptible population is a promising concept to consider in such regions. This would enable a quicker recovery of the efficacy and usage of compromised drugs than is available through cessation of their usage. Anthelmintic chemicals are the main – if not almost exclusive – tool for controlling parasites in pasturing livestock systems (Caracostantogolo et al., 2013). Of course, once efficacy is recovered it is critical then to implement strategies to maintain the newly susceptible population for as long as possible.

Armour (1980) pointed out that knowledge of parasite epidemiology, patterns of pasture contamination and infectivity and the ecology of free-living stages are crucial for establishing sustainable alternatives for parasite control. One such alternative is to change an existing parasite population through the establishment of new susceptible refugia. Seasonal variations in parasite populations in the host and on pasture, i.e. the host-parasite and parasite-environment relationships respectively, are crucial understandings to apply and leverage when attempting such a change. Establishing a new population is arguably easier if a smaller existing resistant refugium is present at the time of implementing the strategy.

In the Humid Pampa region of Argentina, the cattle production system starts with the cow-calf stage, which usually takes place on pastured areas especially used for this purpose and where application of anthelmintic treatments is rare or not at all. This is followed by the feeding/fattening stage where recently weaned and treated calves are placed on different and better pastures from autumn to early spring. Here they are exposed to the low level of infectivity (small refugia) that has survived the summer conditions following the previous production cycle (Fiel et al., 2012; Steffan et al., 2012).

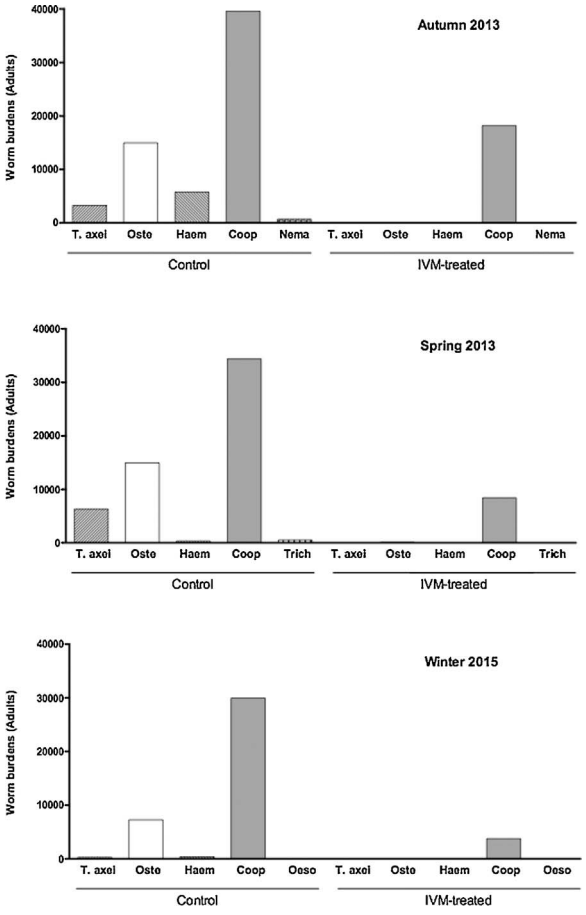

Fig. 2. Geometric means of adult nematode counts at necropsy on the three occasions when controlled efficacy tests (CET) were conducted. T. axei: *Trichostrongylus axei*, Oste: *Ostertagia*, Haem: *Haemonchus*, Coop: *Cooperia*, Nema: *Nematodirus*, Trich: *Trichostrongylus* spp. (small intestine), Oeso: *Oesophagostomum*.

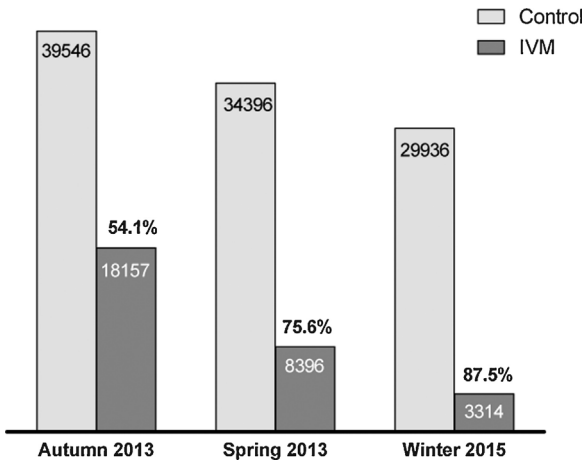

Fig. 3. Comparative evolution of the recovery of absolute efficacy, measured by CET, of ivermectin on *C. oncophora* and *C. punctata*. The proportional relations between *C. oncophora* and *C. punctata* were: Autumn 2013, 80/20; spring 2013, 75/24 (1% *C. mcmasteri*); winter 2015, 65/35.

This management approach could offer a useful and practical opportunity to introduce susceptible populations directly from the cow-calf stage – always confirming first the absence of AR – and to start to reduce and eventually correct the resistance level present. Removing the treatment given to calves at weaning would allow these calves to 'seed' the pasture with susceptible nematodes and initiate the re-

establishment of susceptible refugia. This approach would result in pastures being quickly contaminated with susceptible parasite eggs, instead of having to wait for some 3 weeks before calves start shedding eggs following the usage of artificial infections with susceptible L3. This and other alternative strategies will be considered and assessed in future trials.

The main objective of the present study was to explore the possibility that an ivermectin-resistant population could be replaced by a susceptible one in a cattle production system. The results seen demonstrated that it was possible to increase the absolute efficacy of ivermectin from around 50% to levels close to 90% in just two production cycles. These results indicate that this alternative deserves to be considered further and encourages the continuation of this line of research. Future aspects to explore should start to address potential improvements in the process efficiency, especially the method of seeding (either artificially or naturally), as well as the supportive diagnostics that can help with understanding the dose timing and the type and level of dose required each year. Farms where reversion has been established also provide an opportunity for the evaluation of approaches designed to slow the development of resistance.

5. Conclusions

The experimental infections at weaning with ivermectin-susceptible *Cooperia* spp. were successful. Appropriately high levels of FEC as well as the coprocultures and pasture infectivity results confirmed the establishment of a new refugia dominated by the inoculated *Cooperia* spp. Both the FECRT and CET results demonstrated a progressive and meaningful return of ivermectin efficacy over the two years of the study. These observations confirm the potential for a resistant parasite population to be replaced by a susceptible one through appropriate inoculation of susceptible of *Cooperia* spp. Knowledge and exploitation of parasite ecology and epidemiology is a key component of succeeding with this potential and the results of this study encourages the continuation of this line of research towards finding a practical alternative for the recovery of ivermectin efficacy in grazing production systems.

Conflict of interest statement

The authors declare no conflict of interest and disclose no financial relationship with people or organizations that could bias this work.

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References

Anziani, O.S., Fiel, C.A., 2015. Resistencia a los antihelmínticos en nematodos que parasitan a los rumiantes en la Argentina. Rev. Investig. Agropecu. 41, 34–46.
Anziani, O.S., Zimmermann, G., Guglielmone, A., Vazquez, R., Suárez, V., 2001. Avermectin resistance in Cooperia pectinata in cattle in Argentina. Vet. Rec. 149, 58–59. http://dx.doi.org/10.1136/vr.149.2.58.
Armour, J., 1980. The epidemiology of helminth disease in farm animals. Vet. Parasitol. 6, 7–46.
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. http://dx.doi.org/10.1016/j.vetpar.2011.11.057.
Bird, J., Shulaw, W.P., Pope, W.F., Bremer, C.a., 2001. Control of anthelmintic resistant endoparasites in a commercial sheep flock through parasite community replacement. Vet. Parasitol. 97, 219–225. http://dx.doi.org/10.1016/S0304-4017(01)00406-X.
Caracostantogolo, J., Anziani, O., Romero, J., Suárez, V., Fiel, C., 2013. Resistencia a los antihelmínticos en Argentina. In: Fiel, C., Nari, A. (Eds.), Enfermedades Parasitarias de Importancia Clínica Y Productiva En Rumiantes. Fundamentos Epidemiológicos Para Su Diagnóstico Y Control. Editorial Hemisferio Sur, Montevideo, pp. 255–282.
Coles, G.C., Bauer, C., Borgsteede, F.H.M., Geerts, S., Klei, T.R., Taylor, M.A., Waller, P.J., 1992. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. Vet. Parasitol. 44, 35–44. http://dx.doi.org/10.1016/0304-4017(92)90141-U.
Cristel, S., Fiel, C., Anziani, O., Descarga, C., Cetrá, B., Romero, J., Fernández, S., Entrocasso, C., Lloberas, M., Medus, D., Steffan, P., 2017. Anthelmintic resistance in grazing beef cattle in central and northeastern areas of Argentina—an update. Vet. Parasitol. Reg. Stud. Reports 9, 25–28. http://dx.doi.org/10.1016/j.vprsr.2017.04.003.
De Graef, J., 2013. Detection and Mechanisms of Macrocyclic Lactone Resistance in the Bovine Nematode Cooperia Oncophora. Ghent University.
Fiel, C., Steffan, P., Ferreyra, D., 2011. Diagnóstico de las parasitosis más frecuentes de los rumiantes: Técnicas de diagnóstico e interpretación de resultados. Abad Benjamín, Buenos Aires.
Fiel, C.A., Fernández, A.S., Rodríguez, E.M., Fusé, L.A., Steffan, P.E., 2012. Observations on the free-living stages of cattle gastrointestinal nematodes. Vet. Parasitol. 187, 217–226. http://dx.doi.org/10.1016/j.vetpar.2012.01.011.
Fiel, C., Steffan, P., Entrocasso, C., 2013. Epidemiología e impacto productivo de nematodos en la Pampa Húmeda. In: Fiel, C., Nari, A. (Eds.), Enfermedades Parasitarias De Importancia Clínica Y Productiva En Rumiantes. Fundamentos Epidemiológicos Para Su Diagnóstico Y Control. Editorial Hemisferio Sur, Montevideo, pp. 29–58.
Fiel, C., Steffan, P., Bernat, G., Riva, E., 2016. The control of trichostrongyle infections in grazing cattle of Argentina in a context of multiple anthelmintic resistance. J. Vet. Med. Res. 3, 1–6.
Henriksen, S.A., Korsholm, M., 1983. A method for culture and recovery of gastrointestinal strongyle larvae. Nord. Veterinærmed. 35, 429–430.
Kaplan, R.M., 2004. Drug resistance in nematodes of veterinary importance: a status report. Trends Parasitol. 20, 477–481. http://dx.doi.org/10.1016/j.pt.2004.08.001.
Knox, M.R., Besier, R.B., Le Jambre, L.F., 2012a. Foreword. Vet. Parasitol. 186, 1. http://dx.doi.org/10.1016/j.vetpar.2011.11.040.
Knox, M.R., Besier, R.B., Le Jambre, L.F., Kaplan, R.M., Torres-Acosta, J.F.J., Miller, J., Sutherland, I., 2012b. Novel approaches for the control of helminth parasites of livestock VI: summary of discussions and conclusions. Vet. Parasitol. 186, 143–149. http://dx.doi.org/10.1016/j.vetpar.2011.11.054.
Kotze, A.C., Hunt, P.W., Skuce, P., von Samson-Himmelstjerna, G., Martin, R.J., Sager, H., Krücken, J., Hodgkinson, J., Lespine, A., Jex, A.R., Gilleard, J.S., Beech, R.N., Wolstenholme, A.J., Demeler, J., Robertson, A.P., Charvet, C.L., Neveu, C., Kaminsky, R., Rufener, L., Alberich, M., Menez, C., Prichard, R.K., 2014. Recent advances in candidate-gene and whole-genome approaches to the discovery of anthelmintic resistance markers and the description of drug/receptor interactions. Int. J. Parasitol. Drugs Drug Resist. 4, 164–184. http://dx.doi.org/10.1016/j.ijpddr.2014.07.007.
Leathwick, D.M., Besier, R.B., 2014. The management of anthelmintic resistance in grazing ruminants in Australasia-strategies and experiences. Vet. Parasitol. 204, 44–54. http://dx.doi.org/10.1016/j.vetpar.2013.12.022.
Martin, P., Anderson, N., Jarrett, R., 1989. Detecting benzimidazole resistance with faecal egg count reduction tests and in vitro assays. Aust. Vet. J. 66, 236–240.
Moussavou-Boussougou, M.-N., Silvestre, a, Cortet, J., Sauve, C., Cabaret, J., 2007. Substitution of benzimidazole-resistant nematodes for susceptible nematodes in grazing lambs. Parasitology 134, 553–560. http://dx.doi.org/10.1017/S0031182006001697.
Muchiut, S., Fernández, S., Steffan, P., Lloberas, M., Luque, S., Cardozo, P., Bernat, G., Riva, E., Fiel, C., 2016. The recovery of fenbendazole efficacy on *Haemonchus contortus* by refugia management and worm population replacement Haemonchus Control group (n = 6) Year 1 Treated group (n = 6) Initial efficacy Control group (n = 6) Year 2 Treated group (n = 6) F. In: 8th Novel Approaches to the Control of Helminth Parasites of Livestock. Belem.
Niec, R., 1968. Cultivo e identificación de larvas infectantes de nematodes gastrointestinales del bovino y ovino, Manual técnico Nro. 3. INTA CICV Castelar, Castelar.
Presidente, P.J.A., 1985. Methods for detection of resistance to anthelmintics. In: Anderson, N., Waller, P.J. (Eds.), Resistance in Nematodes to Anthelmintic Drugs. CSIRO, Division of Animal Health and Australian Wool Corporation, New South Wales, pp. 13–27.
Steffan, P.E., Fiel, C.A., 1986. Bioecología de los nematodes gastrointestinales de los bovinos. Rev. Argentina Prod. Anim. 6, 139–140.
Steffan, P.E., Fiel, C.A., Saumell, C.A., Fusé, L.A., Iglesias, L.E., 2005. El uso de antihelmínticos en los programas de control y riesgo potencial de resistencia. Resistencia a Los Antiparasitarios Internos En Argentina. FAO Producción y Sanidad Animal, Rome, pp. 85–92.
Steffan, P., Fiel, C., Ferreyra, D., 2012. Endoparasitosis más frecuentes de los rumiantes en sistemas pastoriles de producción: Aspectos básicos de consulta rápida. IPCVA, Buenos Aires.
Steffan, P.E., Fiel, C.A., Entrocasso, C., Salada, D., 2013. Control de nematodos en bovinos. In: Fiel, C.A., Nari, A. (Eds.), Enfermedades Parasitarias de Importancia Clínica Y Productiva En Rumiantes. Fundamentos Epidemiológicos Para Su Diagnóstico Y Control. Editorial Hemisferio Sur, pp. 175–200.
Sutherland, I.A., Leathwick, D.M., 2011. Anthelmintic resistance in nematode parasites of cattle: a global issue? Trends Parasitol. 27, 176–181. http://dx.doi.org/10.1016/j.pt.2010.11.008.
Taylor, M.A., Hunt, K.R., Goodyear, K.L., 2002. Anthelmintic resistance detection methods. Vet. Parasitol. 103, 183–194. http://dx.doi.org/10.1016/S0304-4017(01)00604-5.
Van Wyk, J., Schalkwyk, P., 1990. A novel approach to the control of anthelmintic-resistant Haemonchus contortus in sheep. Vet. Parasitol. 35, 61–69.
Wolstenholme, A.J., Fairweather, I., Prichard, R., Von Samson-Himmelstjerna, G., Sangster, N.C., 2004. Drug resistance in veterinary helminths. Trends Parasitol. 20, 469–476. http://dx.doi.org/10.1016/j.pt.2004.07.010.