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Vincent Leignel, Anne Silvestre, Jean-François Humbert, Jacques Cabaret

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V. Leignel[a], A. Silvestre[b], J.F. Humbert [c] and J. Cabaret[b]
[a] Université du Maine, Laboratoire Mer-Molécules-Santé, F-72085 Le Mans, France
[b] INRA, UR1282 Infectiologie Animale et Santé Publique, F-37380 Nouzilly, France
[c] INRA UMR Bioemco, Site de l'ENS, 75005 Paris, France
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Abstract
The evolution of benzimidazoles (BZ) resistance in *Teladorsagia circumcincta* was investigated in a controlled trial with lambs, submitted to different treatment regimens. Four paddocks were seeded with a *T. circumcincta* strain constituted by 25% of BZ-resistant nematodes. Ten permanent lambs were allocated to each paddock, from April to November in order to renew the contamination of pasture. Monthly, three tracer lambs were allocated in each paddock. BZ-resistant nematode frequency was determined (PCR diagnosis). The faecal egg count reduction test (permanent lambs) and the number of nematodes in lambs were also determined (permanent and tracer lambs). Four different regimens of treatments were performed: control, levamisole (a non-BZ drug), fenbendazole (a BZ drug), and an alternation of levamisole and fenbendazole every second treatment. The same protocol was repeated on two consecutive grazing seasons, increasing the number of treatments (3 in first year and 5 in second year). The proportions of BZ-resistant nematodes did not change during all the study in both the control and the levamisole paddocks, supporting an equal global fitness of BZ-resistant and susceptible nematodes. Thus, no reversion of BZ resistance is to be expected. In the alternated drug group and in the BZ treated group, BZ-resistant nematodes increased from 25% to 47% and to 78%, respectively. BZ resistance increased proportionally to the selective pressure (number of BZ treatments). The drug alternation is not a good solution to delay importantly the evolution of resistance when more than 25% of nematodes are BZ-resistant. This study is the first evaluation of BZ-resistance evolution (using individual genotyping) in controlled conditions. It showed that when a monogenic anthelmintic resistance is established at 25% in a sexually reproducing nematode population, it seems to be impossible to prevent its increase even when using limited number of BZ treatments.
Keywords: Treatment alternation; Anthelmintic; Levamisole; Benzimidazoles; Nematode; *Teladorsagia*

1. Introduction

The resistance against therapeutical products (antibiotic, anthelmintic, and insecticide) is actually one of the major contemporary challenges for health maintenance. The anthelmintic resistance of nematodes constitutes a problem in small ruminants and other herbivores in most regions of the world ([Chartier et al., 2001] and [Kaplan, 2004]). Rare new molecules are found such as cyclooctadepsipeptides (Von Samson-Himmelstjerna et al., 2005), or amino-aceto-nitrile derivatives (Prichard and Geary, 2008) and they are not marketed for large animals, for which there has not been a new class of anthelmintics introduced in marketplace in almost 25 years (Kaplan, 2004). We must rely in most places on three distinct anthelmintic groups with different modes of action: the benzimidazoles (BZ)/probenzimidazoles, the imidazothiazoles/tetrahydropyrimidines and the macrocyclic lactones (Fraser et al., 2006). Unfortunately, the resistance emergency exists for these three drugs ([Sangster and Gills, 1999], [Sangster, 2001], [Kaplan, 2004] and [Brady and Nichols, 2009]). The BZ remain widely used in herbivores throughout the world since they are cheap and liberate few harmful residues in the environment. BZ-resistant nematodes are efficiently controlled by imidazothiazoles or macrocyclic lactones ([Overend et al., 1994] and [Waruiru et al., 1996]). To maintain the BZ drug efficacy and to control the evolution of resistance, the alternation of drugs or a simultaneous use of minimum two molecules had been proposed ([Donald et al., 1980], [Prichard et al., 1980], [Dobson et al., 1987], [Waller et al., 1989], [Uhlinger and Kristula, 1992], [Coles, 1994], [McKenna et al., 1996] and [Andrews, 2000]). Formulations permitting the simultaneous use of two or three molecules from the existing three main groups of anthelmintics are available in countries such as Australia (Q-drench®, Triton®, etc.), New Zealand or Uruguay among others. This solution is not sustainable from the residues, environment and efficacy point of views. An alternation of BZ and another drug has been proposed ([Waller et al., 1989], [McKenna, 1990] and [Grimshaw et al., 1996]). Some studies had tested the anthelmintic alternation in experimental conditions, at a time where the genetic main mechanisms of resistance to BZ was not known. Furthermore, evaluation of such alternation should be made in natural conditions since only the parasitic stages (larvae and adult) are submitted to anthelmintic pressures (in a lesser extent arrested larvae) and the free-living stages on pastures can be compared to a sub-population not submitted to selective pressures (in "refuge": [Gaba et al., 2006a] and [Kenyon et al., 2009]), that would preserve the anthelmintic susceptible alleles.

We studied the evolution of BZ resistance in a population of *Teladorsagia circumcincta* (Nematoda, Trichostrongylidae), one of the most frequent trichostrongyle in the temperate climatic zone. Its life cycle presents a free-living phase (larvae stages) on pastures and a parasitic phase in the small ruminant (adult stage). Although several mutations were identified in β-tubulin gene ([Silvestre and Cabaret, 2002] and [Ghisi et al., 2007]), BZ

resistance is mainly conferred by a recessive point mutation in isotype 1 of the β tubulin gene of *T. circumcincta* (Elard and Humbert, 1999) as in most digestive-tract strongyles of ruminants. One partially resistant isolate was seeded on four paddocks and each flock on each paddock has a particular anthelmintic regimen in order to evaluate putative reversion (untreated control), interaction with levamisole, and the influence of selection pressure with BZ (alternation of treatment of BZ with levamisole or only BZ) in condition of natural reinfection during two grazing seasons.

2. Materials and methods

2.1. Nematode origin and characterisation

The *T. circumcincta* strain was isolated from a French goat farm in 1996 and maintained in laboratory conditions during two generations. This strain was genotyped for the BZ resistance by PCR according to Humbert and Elard (1997). This strain was genotyped in natural conditions (in farm) in 1991 and 1996 (Elard et al., 1999), and before this study. All these estimations showed that this strain harboured 25% of BZ-resistant (homozygote rr) nematodes and 75% of BZ-susceptible nematodes (25% homozygote SS and 50% heterozygote rS) (Elard et al., 1999). Twenty-five percent of BZ-resistant nematodes correspond to the lowest level of resistance detected by phenotypic measures (Martin et al., 1989). The *T. circumcincta* strain was fully susceptible to levamisole.

2.2. Experimental conditions

The experiment was conducted under temperate climate in Nouzilly, in western France during two consecutive years. Rainfalls were 861 and 1032 mm during the two consecutive grazing seasons, respectively, the latter being over average. Four adjacent paddocks (7000 m^2 of new pasture constituted of rye-grass and white clover) were seeded with the above described *T. circumcincta* strain. The paddocks were first seeded with *T. circumcincta* from September to October before the first grazing year with 20 lambs previously infected with 8000 infective larvae (L3). In November, three pairs of these lambs were either treated with levamisole, fenbendazole or remained untreated. The efficacies were 99.5% and 83.0%, respectively, for levamisole and fenbendazole. In March, the future permanent lambs (naive Romane breed) were infected with 4000 L3 of the same isolate so that they were excreting eggs at turn out. Ten permanent lambs were grazed during all the grazing season (mid-April to mid-November) on each paddock. Permanent lambs present in paddock 1 were non-treated controls and those on paddock 2 were treated with levamisole (efficient against BZ-resistant nematodes, Anthelsol®). Permanent lambs from paddock 3 were alternatively treated with levamisole (in August during first year, June and September during second year) or a benzimidazole, fenbendazole (Panacur®, in May and October of first year and in May, July and October in second year) (Fig. 1). Permanent lambs from paddock 4 were treated with fenbendazole. Permanent lambs were treated at the manufacturer recommended ovine dose. We thus realized different selective processes on the populations according to the anthelmintic regimen (Fig. 1). Both phenotypic and genotypic measures of BZ resistance were achieved. Treatment efficacy was determined by the faecal egg count reduction test (FECRT) for the two grazing seasons, on three occasions (May, September and October for the first year; June, September and October for second year). The estimation of eggs per gram (EPG) was realized the day of the treatment and 11 days after treatment and a FECRT was calculated according to (Dash et al., 1988), as this formula takes into account the possible evolution of egg output in control group. Dash et al., 1988 formula relies on before and after treatment evaluation in treated and control hosts: FECR = 100 $(1 - [T_2/T_1]/[C_1/C_2])$ where T_2 and T_1 are pre- and post-treatment arithmetic means of the EPG in treated groups and C_1 and C_2 are pre- and post-treatment arithmetic means of the EPG in the controls. Phenotypic resistance can be measured as 1 − FECR.

Fig. 1. Anthelmintic treatment program applied in the flocks grazing the four paddocks. BZ, benzimidazoles; Lev, levamisole.

At the end of each grazing season, all permanent lambs were slaughtered and nematodes counted on an aliquot as indicated in (Gaba et al., 2006b). For each paddock, a minimum 50 nematodes per permanent lamb were genotyped to detect the BZ resistance according to Humbert and Elard (1997). A total of 1249 *T. circumcincta* from these permanent lambs were individually genotyped into BZ-resistant (rr), BZ-susceptible homozygotes (SS) and heterozygotes (rS) for this study. In parallel, three tracer lambs were introduced in each paddock at monthly or bimonthly intervals (July–August) during the two grazing seasons. After one month, each tracer lamb was brought indoors for two weeks and fed on uninfected hay, to let the late ingested larvae mature into adults. The tracer lambs were finally necropsied to collect the parasites. Altogether, 120 tracer lambs were slaughtered, and 5658 *T. circumcincta* (minimum 40 nematodes per lambs) were genotyped to quantify the frequency of BZ-resistant genotypes.

2.3. Statistical analysis

A general linear model (GLM) was used to compare effects of treatment regimen on egg output, nematode burden and frequency of BZ-resistant nematodes in different paddocks. The GLM use of a hierarchical regression analysis technique allows greater flexibility than usual analysis of variance by allowing one to combine quantitative and categorical variable and to control for

covariates. Thus a model, $y = a_1 \times x_1 + a_2 \times x_2 + \quad + a_i x_i$ + error is evaluated, where y is the parameter we want to evaluate (e.g., EPG) in relation to other experimental factors (x_1 to x_i, e.g. paddock, season, year of experiment). The value of the model is expressed by a R^2 value and a level of statistical significance p. When the model was significant estimated marginal averages were calculated. These averages were adjusted for the covariates (season and

year of experiment for tracer lambs, and year for permanent lambs). The data did not follow a Gaussian distribution, and were neperian logarithm transformed (log + 1). Post hoc significance was established with Newman–Keuls test. Calculations were performed using Simstat software (Péladeau and Lacouture, 1993).

3. Results

3.1. Dynamic of infection based on permanent lamb egg output and adult nematode burden in tracer lambs

Nematode burden of permanent lambs illustrate the effects of repeated treatments on nematode establishment after one year of infection (Table 1). The number of nematodes and the number of arrested larvae per permanent lamb were not significantly different between paddocks during the first and the second grazing season (Table 1). Arrested larvae were three- to fourfold higher in second season in comparison with the first, in all four paddocks. These data give a good picture of infection at the end of grazing season but do not inform on the parasitic pressure all along the grazing season. Conversely the average EPG (Table 1) give further information on parasitic pressure: it was highest in the control paddock in the second grazing season, as observed from nematode burdens.

Table 1.
Average, standard deviation (SD) and range of adult nematodes and arrested larvae (L4) per permanent lamb necropsied in the two consecutive seasons for the four paddocks.

EPG	**Paddock 1 (control)**	**Paddock 2 (levamisole treatment)**	**Paddock 3 (alternation of treatment)**	**Paddock 4 (fenbendazole treatment)**				
First year								
Average	168[a*]	129[a]	141[a]	145[a]				
SD	86	64	48	69				
Range	25–260	39–229	30–171	28–229				
Second year								
Average	98[a]	58[b]	55[b]	72[b]				
SD	50	31	29	41				
Range	41–193	13–107	28–113	34–107				
Nematodes	**Adults**	**L4**	**Adults**	**L4**	**Adults**	**L4**	**Adults**	**L4**
First year								
Average	2188[a]	4483[a]	1533[a]	2860[a]	1974[a]	8473[a]	3249[a]	5195[a]
SD	2020	3660	1243	3741	1174	6705	4186	5045
Range	128–7938	0–11,900	95–4592	200–11,400	58–3598	0–23,700	260–13,075	508–17,833
Second year								
Average	3411[a]	11,636[b]	2324[a]	9747[b]	1524[a]	10,464[b]	2453[a]	14,880[b]
SD	2611	17,522	978	13,376	665	16,558	2108	27,701

EPG	Paddock 1 (control)	Paddock 2 (levamisole treatment)	Paddock 3 (alternation of treatment)	Paddock 4 (fenbendazole treatment)				
Range	1202–9768	0–53,170	845–3710	0–32,310	634–2813	0–48,035	60–6872	0–71,570
Estimated marginal average**	2798[a]	7714[b]	1928[a]	6303[b]	1749[a]	8863[b]	2851[a]	10,037[b]

* Similar letters in a column indicates that no significant difference was shown between paddocks and in a line, between years, using GLM.
** GLM with year and paddock as factors.

During summer, an increase of EPG was observed in all paddocks for the two years (Fig. 2). In first grazing season, the average egg outputs per permanent lambs in the control paddock (paddock 1) was higher than EPG observed in all treated paddocks (Table 1), indicating that even when 25% of nematodes are BZ-resistant, BZ treatment is useful to reduce egg outputs and pasture contamination. In second grazing season, average egg outputs were twice higher than in first year in paddock 4 (benzimidazole treatment) whereas other paddocks presented similar egg outputs (Table 1). The control paddock had globally higher egg outputs than other paddocks.

Fig. 2. Evolution of average egg outputs during the two consecutive grazing seasons in permanent lambs of the four paddocks (1: control, 2: levamisole, 3: levamisole and benzimidazole, 4: benzimidazole). Figures correspond to estimated marginal averages, estimated from GLM: R^2 = 0.33, p = 0.001; significant covariates: paddock, year, month, and interaction between paddock, year and month.

Nematode burdens observed in tracer lambs illustrate the infection level of each paddock (Fig. 3 and Table 2). The strongest increase of nematode burden was observed during the spring of first year and the summer of second year, in all paddocks. The number of nematodes in tracer lambs was higher in the paddock 1 (control group) reaching an average of 7274 and 8400 nematodes per lamb in the two consecutive grazing seasons, respectively. No significant difference was observed between the paddocks 2–4 (treated groups) in first grazing season. Conversely, in second year, the number of nematodes in tracer lambs was influenced by the anthelmintic regimen administered to permanent lambs. In the paddocks 2 (levamisole treatment) and 3 (alternating treatment), nematode populations were limited to 5000 nematodes in average (maximum observed 9739 nematodes); but in paddock 4 (benzimidazole treatment) nematode population reached 6800 nematodes in average, reaching a maximum observed around 14,600 nematodes, in spite of fenbendazole treatment (Fig. 3 and Table 2).

Fig. 3. Evolution of average nematode burden during the two consecutive grazing seasons in tracer lambs of the four paddocks (1: control, 2: levamisole, 3: levamisole and benzimidazole, 4: benzimidazole). Figures correspond to estimated marginal averages, estimated from GLM: R^2 = 0.72, p = 0.001; significant covariates: paddock, year, month, and interaction between paddock, year and month.

Table 2.
Average, standard deviation (SD) adult nematodes per tracer lamb necropsied in the two consecutive grazing seasons for the four paddocks.

	Paddock 1 (control)	Paddock 2 (levamisole treatment)	Paddock 3 (alternative treatment)	Paddock 4 (fenbendazole treatment)
First grazing season				
Average	7274	4122	4065	3493
SD	8390	3425	3644	2391

	Paddock 1 (control)	Paddock 2 (levamisole treatment)	Paddock 3 (alternative treatment)	Paddock 4 (fenbendazole treatment)
Range	700–21,438	587–9602	297–9706	457–6296
Second grazing season				
Average	8400	3972	5473	6798
SD	8615	3732	3910	6788
Range	386–21,132	309–9739	800–9499	422–14,599
Estimated marginal average[*]	7758[a**]	4093[b]	4376[b]	5068[b]

[*] GLM with year, period and paddock as factors, $p = 0.0001$
[**] Similar letters in a line indicates that no significant difference was shown between paddocks using GLM.

3.2. Evolution of resistance in relationship to benzimidazole selective pressure

A phenotypic and a genotypic measure of BZ anthelmintic resistance were achieved with a FECRT and a PCR diagnosis, respectively (Table 3). Conversely to genotyping, the FECR data presented a less clear evolution of resistance. In paddock 3, the efficacy of BZ drug was unchanged in first grazing season (switching from 67% to 69%), although the frequency of BZ-resistant genotypes increased moderately from 22.9% to 31.9%. In second grazing season, the efficacy of BZ drug switched dramatically from 89% to 19%, although the frequency of BZ-resistant genotypes was unchanged (44.8% to 47%). In paddock 4 (benzimidazole treatment), the BZ efficacy switched from 69% to 43%, corresponding to the increase of the frequency of BZ-resistant genotypes from 21.4% to 52.9%. Similarly in second year, BZ efficacy was maintained around 65% and 27% and the frequency of BZ-resistant genotypes increased from 60% to 78%. In paddock 2 (levamisole treatment), the FECRT indicated an important variability of the levamisole efficacy in two consecutive grazing seasons (ranged between 16% and 91%) although the *T. circumcincta* strain is fully levamisole susceptible (Table 3). BZ FECRT evaluated with Dash et al. formula was significantly related to the percentage of r allele ($r_s = -060$; $p = 0.046$ on data of paddocks 3 and 4).

Table 3.
Benzimidazole resistance evaluation (frequency of BZ r allele, frequency of BZ rr nematodes, FECRT) in tracer lambs and in permanent lambs, along the two grazing season experiment. Numbers between brackets correspond to standard deviation. ND: not determined.

		First grazing season						Second grazing season				
		Tracer lambs					Permanent	Tracer lambs				Permanent
		May	June	July–August	September	October	October	June	July–August	September	October–November	November
Paddock 1 Control	% rr nematode	26.2 (4.6)	27.1 (3.9)	22.9 (5.1)	25.5 (10.9)	33.6 (1.7)	27.9 (5.5)	32.8 (12)	26 (13.9)	21.3 (5.5)	16.3 (3.3)	22 (6.4)
	EPG	213[a]	ND	ND	291	92	ND	99	ND	144	150	ND

		First grazing season						Second grazing season				
		Tracer lambs					Permanent	Tracer lambs				Permanent
		May	June	July—August	September	October	October	June	July—August	September	October—November	November
Paddock 2 Levamisole	% rr nematode	22.4 (1.5)	24.5 (4.7)	28.9 (10.6)	32.9 (6.4)	36.6 (7.9)	26 (9.9)	34.7 (6)	33.2 (6.9)	28 (17.1)	28.3 (8.3)	20 (2)
	FECRT	59%[b]	ND	ND	80%	52%	ND	84%	ND	91%	16%	ND
Paddock 3 Levamisole/fenbendazole	% rr nematode	22.9 (3.4)	25.6 (4.9)	44.3 (9.1)	42.7 (2.2)	31.9 (5.8)	53 (6.6)	44.8 (7.4)	31.3 (2.8)	45.1 (9.1)	47 (6.8)	54.3 (13.1)
	FECRT	67%	ND	ND	85%	69%	ND	89%	ND	64%	19%	ND
Paddock 4 Fenbendazole	% rr nematode	21.4 (6.5)	34.9 (2.6)	51.8 (0.6)	41.5 (8.9)	52.9 (6.2)	60.3 (19.6)	60.4 (10.4)	82.7 (5.4)	80.8 (3.4)	78.1 (3.1)	89.5 (4.6)
	FECRT	69%	ND	ND	70%	43%	ND	65%		67%	27%	ND
% rr significance		1 = 2 = 3 = 4[c]									1 = 2 < 3 < 4	
FECRT significance		1 > 2, 3, 4			1 > 2, 3, 4	1 > 2, 3, 4		1 > 2, 3, 4		1 < 2, 3, 4	1 = 2 = 3 = 4	

Full-size table

[a] Estimated EPG.

[b] FEC reduction using Dash et al. formula.

[c] Significance of difference between paddocks (e.g., 1 > 1, 2, 3, 4: control (paddock 1) has higher EPG than treated groups).

In all four paddocks, BZ resistance was measured monthly (bimonthly in July–August) by the PCR diagnosis on adult nematodes, collected after necropsy of tracer lambs. No significant evolution of the frequency of BZ-resistant genotypes was observed in the paddocks 1 (control) and 2 (after 8 levamisole treatments) during the second grazing season. The frequency of rr was well estimated by a GLM (R^2 = 0.87, p = 0.001). The significant variables in model were paddock, year, month, and interaction between paddock, year and month. The significant differences between paddocks were evaluated with Newman–Keuls (see the legend in Table 3). Frequency of BZ-resistant nematodes was maintained around 25–30%. In paddock 3 where permanent lambs were treated with an alternating anthelmintic regimen (levamisole or fenbendazole), the frequency of BZ-resistant genotypes increased from 23% to 47% after 5 BZ and 3 levamisole treatments, during the two grazing seasons. The strongest increase of the frequency of BZ-resistant genotypes was observed in the paddock 4, where only fenbendazole treatments were realized: BZ-resistant genotypes increased from 21% to 78% after 8 BZ treatments (Table 3). The frequency of BZ-resistant genotypes in permanent lambs, at the end of the grazing season, was 28% (paddock 1), 26% (paddock 2), 53% (paddock 3) and 61% (paddock 4) in first year. After the second grazing season, this frequency was unchanged in paddock 1 (22%), in paddock 2 (20%) and in paddock 3 (54%), but in paddock 4 the frequency of BZ-resistant genotypes reached 89%. In paddock 2 (levamisole treated group), levamisole treatment administered in October from second grazing year was inefficient: 16% (Table 3). This is consistent with the known inefficacy of levamisole against arrested L4 larvae, which represent 93% of the whole nematode burden in paddock 2 (Table 2).

4. Discussion

Our main objective was to investigate the evolution of BZ resistance when nematodes were maintained under different treatment regimens. In our experimental design, the grazing season ranged from April–May to November, the stocking rate was 16 lambs/ha, the number of anthelmintic treatments ranged from 3 (in first year) to 5 (in second year), we used fenbendazole (a benzimidazole) and levamisole (an imidazothiazole) anthelmintics. Although our experimental design with one single species was simplified compared to ordinary farm where lambs are infected with a community of nematodes, this simplification should not affect the qualitative findings on control and resistance reported here.

Few studies were conducted on drug combination efficiency, and anthelmintic resistance was monitored by phenotypic methods (FECRT and *in vitro* lethal dose on eggs, see [Cooper et al., 1996], [Maingi et al., 2002], [Wrigley et al., 2006] and [Sutherland et al., 2008]). Drug alternation studies were also rare (Uhlinger and Kristula, 1992). In the present study, we monitored precisely evolution of BZ-resistant gene frequency by individual genotyping of nematodes. We used FECRT to estimate phenotypic efficacy and subsequent impact of treatments on pastures contamination. For both BZ and levamisole, FECRT yielded very different estimates of efficacy. Although not related to anthelmintic resistance, poor efficacy of both drugs has been recorded previously, in part related to the presence of *T. circumcincta* inhibited larvae which are less susceptible to either imidazothiazoles ([Walley, 1966], [McKenna, 1974] and [Williams, 1991]) or BZ (Cabaret et al., 1979) to a lesser extent, as observed at the end of each grazing season. The irregular efficiency of FECRT to detect resistance has been questioned (Humbert et al., 2001) and we concentrated on the frequency of BZ-resistant nematodes to express BZ resistance.

In paddock 1 (control group) and paddock 2 (levamisole group), final frequencies of BZ-resistant and BZ-susceptible nematodes in permanent lambs were unchanged at the end of the study (Table 3). However it seems that autumn period is unfavourable to the survival of rr larvae since there is a decrease in percentages of rr in control paddock and absence of expected increase of rr in the paddocks 3 and 4 (Table 3). The latter is in accordance with the findings of Elard et al. (1998) in laboratory conditions (parameters: prime-infection of naive lambs, culture from eggs to larvae at stable temperature 23 °C, storage of infective larvae at 4 °C during more than three months). This supports an equivalent “global fitness” (e.g., including major life traits) of resistant and susceptible nematodes, but some life traits (such as survival of free-living stages to low temperature) may be compensated throughout the life cycle of *T. circumcincta*. This is in accordance with others findings on *Haemonchus contortus* (Melo, 2005), and the better survival of BZ-resistant larvae to dry climate and the better survival of BZ-susceptible larvae to hot temperature. The possible reversion of the BZ resistance should be studied furthermore, exploring differential success in life traits of resistant and susceptible nematodes.

Our results show that alternation of BZ and levamisole succeeded in delaying the selection of BZ resistance at medium term, in comparison with the exclusive BZ treatment regimen: in paddock 3 (alternation of BZ and levamisole), frequency of BZ-resistant nematodes reached 45% at the end of the experiment, corresponding to 5 BZ treatments (and 3 levamisole treatments). In paddock 4, the 5th BZ treatment was done in June from second year, and the frequency of BZ-resistant nematodes was 78%. So, the same number of BZ treatment (here, 5 treatments) selected less rapidly resistant nematodes in paddock 3 than in paddock 4. Nevertheless, the alternating treatment regimen has a short lasting effect (Uhlinger and Kristula, 1992). In paddock 4, the frequency of BZ-resistant nematodes switched from 45% to 80% with only one supplementary BZ treatment (administered in June of second grazing season). So, in all likelihood, the alternating treatment regimen would result in such high BZ-resistance frequency during the subsequent grazing season.

In the present study, lambs were set stocked during the entire grazing season, favouring effects of refuge of free-living stages present on pastures (Van Wyk, 2001). During the early phase of selection, when resistance gene frequency is 0.5%, free-living stages present on pastures may favour the loss of resistance gene by genetic drift (Gaba et al., 2006a). Conversely, the present study shows that refuge is not efficient anymore to delay selection when the frequency of BZ-resistant nematodes reaches high level such as 25% of the population. This study demonstrated that when the BZ-resistant nematodes are present at medium level (25% in the present study) in natural population, no reversion seems to be possible, even if

another anthelmintic molecule without any cross-resistance is used. Refuge is not efficient enough at medium resistance level to allow the loss of resistant alleles by genetic drift (Gaba et al., 2006a). The alternating treatment with levamisole/fenbendazole seemed efficient to control adult nematodes, but it did not allow the control of arrested larvae or the delay of BZ-resistance allele selection. As a conclusion, we may recommend to use BZ until BZ resistance is fully present, under temperate climate. First, even for medium high BZ-resistance frequency, BZ treatment allowed a reduction of both nematode burden and pasture contamination. Second, BZ are broad-spectrum anthelmintics allowing *Dicrocoelium* and pulmonary strongyles control. Third, BZ are efficient against susceptible arrested larvae, conversely to other broad-spectrum anthelmintics ([Andrews, 2000] and [Bartley et al., 2004]). When BZ resistance is observed, drug combination may favour a better nematode control than drugs alone (Albonico et al., 2003) but further work is required to demonstrate a real synergism between drugs (Entrocasso et al., 2008).

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Corresponding author. Tel.: +33 2 43 83 39 04; fax: +33 2 43 83 37 95.

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