FROM ISOLATES TO A SYNTHETIC LABORATORY POPULATION: MAINTENANCE OF VARIABILITY IN THE NEMATODE *HAEMONCHUS CONTORTUS*

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Summary :

Haemonchus contortus isolates were collected from goats of five locations with different climatic characteristics in Guadeloupe archipelago. They were investigated for morphology, morphometrics and allozyme diversity after passage in immunosuppressed lambs using long acting corticoids. The basic aim of the work was to construct a synthetic strain in laboratory conditions which was representative of the isolates. The isolates were only slightly different although climatic conditions were very different. The resemblance of isolates might be due to the practice of goat exchanges between farms or to their insular origin. However the isolate from a smaller island (Les Saintes) was different (mostly on morphometrics) from all the others originating from Guadeloupe main island. The first assemblage in laboratory resulting from the installation from a mixture of the five isolates was not very representative, whereas the next generation (synthetic strain) resembled all the isolates as shown from allozyme study. Female fecundity and length in established synthetic strain were lower than that recorded in isolates, indicating a decrease in fitness, possibly due to the stability of experimental environment. The representativity of the synthetic strain was good but the strain could still evoluate on further passages and should be evaluated on a large number of generations maintained in laboratory.

KEY WORDS : *Haemonchus contortus*, isolates, synthetic strain, allozymes, morphometrics, Guadeloupe.

Résumé : CRÉATION D'UNE POPULATION SYNTHÉTIQUE D'*HAEMONCHUS CONTORTUS* À PARTIR D'ISOLATS ET MAINTIEN DE LA VARIABILITÉ AU LABORATOIRE

*Des isolats d'*Haemonchus contortus *d'origine caprine ont été récoltés sur cinq sites de l'archipel de Guadeloupe, choisis pour leurs caractéristiques climatiques. Ils ont ensuite été maintenus chez des agneaux immunodéprimés au moyen d'un corticoïde à longue action. Des mesures morphologiques, morphométriques et d'alloenzymes ont été réalisées. L'objectif du travail était de construire, en conditions de laboratoire, une souche synthétique qui soit représentative de l'ensemble des isolats. Les isolats présentaient des différences faibles bien que les conditions climatiques soient assez variées. Ces faibles différences peuvent être imputées à l'habitude des éleveurs d'échanger des chèvres ou bien à l'origine insulaire du nématode. L'isolat issu d'une petite île (Les Saintes) était différent cependant des autres isolats de Guadeloupe, surtout par les mesures morphométriques. Le premier assemblage au laboratoire, qui résultait de l'installation des nématodes provenant des cinq isolats, n'était pas très représentatif. La génération suivante (dite souche synthétique) ressemblait toutefois à l'ensemble des isolats d'après l'analyse génétique des alloenzymes. Des différences de fécondité et de longueur des vers femelles ont cependant été enregistrées chez la souche synthétique par rapport aux isolats. Cela pourrait s'expliquer par la stabilité de l'environnement expérimental. La représentativité de la souche synthétique était donc bonne mais pourrait évoluer au cours des passages successifs; elle devrait être évaluée sur un nombre de générations plus important.*

MOTS CLÉS : Haemonchus contortus, *isolats, souche synthétique, alloenzymes, morphométrie, Guadeloupe.*

INTRODUCTION

Haemonchus contortus is the most widespread nematode parasite of sheep and goats in the tropics. Among the *Haemonchus* species, it is the most diversified either morphologically, genetically or ecologically (Jacquiet *et al.*, 1995). Several ecotypes have been described (Das & Whitlock, 1960) and their morph composition might depend upon climatic environment. In some cases, different ecotypes were found at short distances (Mc Kenna, 1971). The Guadeloupe Archipelago islands (Basse Terre, Grande Terre and Les Saintes islands) in a limited geographic range, present a large variety of rainfall regime and breeding management. Differences could then exist between isolates from several sites in these islands. Creole goat is the main parasite host in these islands (Aumont *et al.*, 1996) and breeders frequently exchange kids or goats. This practice favours the building of new parasite assemblages (Cabaret & Gasnier, 1994). One might also deliberately create, for experimental purpose, a new assemblage of isolates in order to create a synthetic strain that could represent the set of variability in the farms. To our knowledge, there is no available information on the building of such a synthetic

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strain. Will this synthetic strain be representative of the original variability? This is a crucial point as the results obtained from such a synthetic strain could be generalised only if the synthetic strain is representative enough of the different isolates. Will this synthetic strain, maintained under laboratory conditions, preserve its variability? This could be questioned as experimental nematode populations are often modified under laboratory conditions (Gasnier *et al.*, 1992; Gasnier & Cabaret, 1998; Chehresa *et al.*, 1997). The purposes of the present work were two-fold: *i)* to assess genetic and morphometric variability of several isolates of *H. contortus* from Guadeloupe Archipelago, *ii)* and to constitute a representative assemblage in order to obtain a synthetic strain. To interpret genetic resemblance we used direct comparison of distance matrix (static interpretation) and a method used in phylogeny (providing a dynamic interpretation of distances).

MATERIALS AND METHODS

Parasite sampling

During the rainy season 1996, faecal samplings were carried out in ten Creole goats in each of four farms in Guadeloupe and one in Les Saintes islands (coded as Ab, Bf, Cp, Pb in Guadeloupe and St in Les Saintes). Each farm was characterised by its location, its rainfall regime and its breeding management (Table I). The identification of third-stage infective larvae (L3) was performed in order to estimate the nematode genera present in these natural isolates (Table I). The digestive-tract strongyles of the four farms from Guadeloupe were described as resistant to benzimidazoles and susceptible to ivermectine (Barré *et al.*, 1997), whereas no information was available in Les Saintes isolate.

Experimental host

Experimental infections were carried out in 4 to 6 months old Black Belly lambs, previously immunosuppressed by triamcinolone acetonide (Kenacort® 80 mg/animal), a long-acting corticosteroid treatment (Suarez *et al.*, 1992). These lambs were reared inside and fed with herbage before infection. As a precaution, they were drenched orally with ivermectine (Oramec®) and with niclosamide (Yomesane®) to eliminate eventual infections with strongyle nematodes and *Moniezia* sp., respectively, one week before the date of experimental infection. They were housed and then fed with hay dried at 60°C during 72 h in order to prevent infection with helminths.

Experimental design

According to the L3 availability, two ewe lambs were infected with 16,400, 8,600, 15,800, 13,900, 11,200 L3, for Ab, Bf, Cp, Pb and St isolates, respectively. One third of doses was given on three occasions at two-

Farms	Location	Climate	Stocking rate (goats/ha)	Irrigated pasture	Type of pasture	No of anthelmintic treatments per year	% of genera based on infective larvae identification (after the first passage in sheep)
St	Other island South of Basse Terre	Marked dry season Annual rainfall of 1,000 mm	Not estimated	No	Unfenced pastures	Not known	81% *Haemonchus* 19% *Trichostrongylus*
Ab	Northern Grande Terre	Marked dry season Annual rainfall of 1,000-1,500 mm	26.9	No	Rotation on less than 4 paddocks	6-12	83% *Haemonchus* 17% *Trichostrongylus*
Bf	Leeward Basse Terre	Marked dry season Annual rainfall of 1,000-1,500 mm	17.6	Yes	Rotation on more than 4 paddocks	12	97% *Haemonchus* 3% *Trichostrongylus*
Cp	Windward Basse Terre	No marked dry season Annual rainfall of 2,000-3,000 mm	34.4	No	Rotation on less than 4 paddocks	6-12	94% *Haemonchus* 6% *Trichostrongylus*
Pb	Windward Basse Terre	No marked dry season Annual rainfall of 2,000-3,000 mm	17.6	No	Roptation on less than 4 paddocks	12	91% *Haemonchus* 9% *Trichostrongylus*

Table I. – Characteristics of farms (location, climate, pastures, anthelminthic treatments, animal breeding and main genera of digestive-tract strongyles).

day intervals. At necropsy 28 days after infection the female adult worms were crushed in order to isolate the eggs that were cultured into infective larvae L3. Then, two ewe lambs were infected (2,000 L3/ewes) with a pool of L3 composed of 25 % Ab, 7 % Bf, 13 % Cp, 1 % Pb and 54 % St. The resulting adult worm infection was named population assemblage (As). 12 ewe lambs were infected daily with 1,500 L3 during 15 days with L3 produced by As. The worm population established from this last infection was named the synthetic strain (Sy). The As and Sy infected lambs were necropsied 42 days after infection. A minimum of 30 males and 30 females of *H. contortus* in each population were collected and stored at – 70°C prior genetic analysis.

PARASITOLOGICAL TECHNIQUES

Infective larvae were produced from faecal eggs extracted and cultured *in vitro* according to Hubert & Kerbœuf (1984). Briefly, eggs extracted from faeces on a 32 µm sieve were separated from residual faecal fibre by centrifugation at 1,800 g for 15 min in chloride sodium solution (density 1.2). Then these eggs were cultured in culture flasks in a buffered Earle media containing died *E. coli*, Amphotericine B and yeast extract. The same culture process was performed for the eggs obtained from crushed female worms.

ANALYSIS OF DIVERSITY

Male characterisation: A discriminant function (DF) combining several measures was used to identify the species of *Haemonchus* (Jacquiet *et al.*, 1997).

$$DF = 0.0016^{*} TL + 0.125^{*} THr + 0.152 THl - 9.97,$$

where TL is the total length of spicule, THr the distance from tip to the hook of the right spicule and THl the distance from the tip to the hook of the left spicule.

If DF is equal or less than 0.63, then the *Haemonchus* species is *H. contortus*, otherwise it corresponds to *H. placei* (DF > 0.63), a usual parasite of bovines that might be found in sheep and particularly goats (Jacquiet *et al.*, 1998). The three spicule measurements (TL, THr and THl) were also used to characterise *H. contortus* isolates and laboratory populations.

Female morphology: The body length, the vulvar morphology and the fecundity (estimated by the number of eggs *in utero*) of each female worm were assessed. The female worms were classified according to their vulvar morphology into linguiform, knobbed and smooth females (Das & Whitlock, 1960). A Fisher exact-test was performed to evaluate the differences in proportions of female morphs between isolates on the one hand, and between laboratory populations (As and Sy) and isolates on the other hand. A General Linear Model implementation of analysis of variance was performed on the morphologic measures of male and female worms (SAS/STAT, 1990). The estimated means presented in all tables are calculated using this general linear model.

Allozyme analysis: The allozymes were studied using isoelectrofocusing on polyacrylamide gel (Brémond, 1987) and staining was as recorded in Gasnier *et al.*, (1992). The following enzymes were studied: phosphoglucomutase (PGM, E.C.5.4.2.2), alcohol dehydrogenase (ADH, E.C.1.1.1.1), malate dehydrogenase (MDH, E.C.1.1.1.37) and mannose-phosphate isomerase (MPI, E.C.5.3.1.8). Phenotypic differences in allozyme banding patterns at a specific locus were used to deduce genotypes of individuals and the number of alleles segregating at the locus. The population was expected to be in a Hardy-Weinberg equilibrium when the worms mate at random and when no selection or immigration process interferes. The indices of Wright (Wright, 1978), F_{is} (coefficient indicating non-random mating within a population), and F_{st} (coefficient indicating non-random mating between populations) were calculated according to the method proposed by Weir & Cockerham (1984) (Genetix Program). Positive F_{is} values indicate a departure from Hardy–Weinberg expectations toward a heterozygote deficiency, and negative values indicate an excess. High F_{st} values correspond to genetically different populations. Negative F_{st} values may artificially appear due to the computation method used by the estimator. The assessment of linkage desequilibrium between loci was performed according to the method proposed by Black & Krafsur (1985). The relationship between isolates, assemblage and synthetic strain was also analysed with Phylip (Felsenstein, 1993) using neighbour joining method (which does not assume the existence of an evolutionary clock) based on Reynolds genetic distances (negative value of natural logarithm ($1-F_{st}$)).

RESULTS

MALE AND FEMALE MORPHOMETRICS

The spicule measures used in the discriminant function indicated that all the male worms were *H. contortus* in the five isolates (Table II) as DF was always lower than 0.63 (data not shown). There was a significant effect of population origin on the total length of spicule (TL) (P = 0.0001). Thus St isolate spicules were longer (TL) than those from the other isolates and the two laboratory populations (P = 0.0001). No difference between populations or isolates was recorded for the ratios (THr/TL) or (THl/TL), coded R1 and R2 respectively (Table II). As for the total length

	No of sheep (no of worms)	Total length (TL) in µm	Distance hook-tip, right spicule (THr) in µm	Distance hook-tip left spicule (THl) in µm	Ratio THr/TL × 100 R1	Ratio THl/TL × 100 R2
Isolates						
Ab	2 (30)	430 *bc*	41.2 *bc*	20.8 *b*	9.61	4.83
Bf	2 (29)	432 *b*	42.8 *a*	20.6 *b*	9.90	4.78
Cp	2 (30)	432 *b*	40.9 *cd*	20.1 *b*	9.48	4.66
Pb	2 (29)	413 *d*	40.2 *c*	20.2 *b*	9.74	4.91
St	2 (30)	446 *a*	42.6 *ab*	22.0 *a*	9.54	4.93
Laboratory populations						
As	2 (40)	430 *bc*	41.6 *abd*	20.7 *b*	9.66	4.80
Sy	8 (144)	425 *c*	40.5 *c*	20.5 *b*	9.53	4.84

Isolates or populations with different letters are significantly different at $P < 0.05$.

Table II. – Estimated means of *H. contortus* male measures of isolates and laboratory populations.

	No of sheep (no of worms)	Knobbed (%)	Smooth (%)	Linguiform (%)	Body length in µm	Eggs *in utero* per female worms
Isolates						
Ab	1 (18)	10	37	53	1,824 *c*	358 *b*
Bf	1 (16)	6	25	69	1,914 *bc*	183 *de*
Cp	1 (19)	26	16	58	1,942 *b*	322 *bc*
Pb	1 (19)	0	47	53	1,951 *b*	278 *cd*
St	1 (19)	31	37	32	2,182 *a*	539 *a*
Laboratory populations						
As	2 (45)	11	51	38	2,085 *a*	224 *d*
Sy	6 (128)	12	43	45	1,838 *c*	161 *e*

Isolates or populations with different letters are different at $P < 0.05$.

Table III. – Vulvar morphs, estimated body length and fecundity means of female *H. contortus* in isolates and laboratory populations.

of spicule, there was a significant effect ($P = 0.0001$) of the population on the total body length of female worms. The St isolate was different from all the isolates and Sy strain for the total length ($P = 0.0001$). The As population was significantly different from Ab and Bf isolates and from Sy strain ($P = 0.0001$) (Table III).

Female morphs and fecundity

The fecundity corrected for female length leads to similar ranking of populations and isolates. The vulvar morph proportions are in Table III. A theoretic assemblage named Asth (40.4 % linguiform, 24.7 % knobbed and 34.4 % smooth) established from the different percentages of each isolate serves as reference to compare the different populations by using a χ^2 test. This theoretic population was significantly different from the As population ($P = 0.0003$ and from the Sy strain ($P = 0.005$). Conversely there was no significant difference between the As and Sy strains. The knobbed morph proportion decreased in As population. St isolate was different from Pb isolate ($P = 0.01$), As ($P = 0.06$) and Sy strain ($P = 0.001$). The St isolate was the most prolific ($P = 0.0001$) (Table III). The Bf isolate, As and Sy populations were the least prolific ($P = 0.001$).

Allozymes

The alcohol dehydrogenase was not polymorphic and phosphoglucomutase was not safely interpretable. Two enzymes (MDH and MPI) out of the four tested were studied because of their polymorphism and the unambiguous interpretation of banding pattern: MDH was a dimeric enzyme and MPI was a monomeric enzyme. Two loci were observed for MDH. Only MDH2 was polymorphic. There was no significant linkage desequilibrium between MDH2 and MPI. All the isolates and the laboratory populations were in Hardy-Weinberg equilibrium for the MDH. Only the St and Pb isolates were in Hardy-Weinberg equilibrium for MPI (Table IV). The lowest F_{st} was found between the Sy strain and all the other populations (Table V). The

		Isolates (no of worms)					Laboratory populations (no of worms)	
		Ab	Bf	Cp	Pb	St	As	Sy
MDH2		(20)	(20)	(30)	(28)	(20)	(30)	(110)
Alleles	1	0.28	0.18	0.20	0.25	0.30	0.20	0.28
	2	0.42	0.62	0.63	0.54	0.47	0.68	0.49
	3	0.30	0.20	0.17	0.21	0.23	0.12	0.23
	H exp*	0.65	0.54	0.53	0.60	0.63	0.48	0.63
	H obs**	0.70	0.50	0.50	0.61	0.65	0.40	0.70
MPI		(20)	(12)	(20)	(19)	(17)	(17)	(85)
Alleles	1	0.03	0.29	0.18	0.05	0.03	0.26	0.18
	2	0.90	0.67	0.75	0.92	0.94	0.71	0.78
	3	0.07	0.04	0.07	0.03	0.03	0.03	0.04
	H exp	0.18	0.47	0.40	0.15	0.11	0.43	0.35
	H obs	0.10	0.17	0.30	0.16	0.12	0.18	0.20

* expected heterozygote frequency.
** observed heterozygote frequency.

Table IV. – Allelic frequencies of malate deshydrogenase (MDH2) and mannose phosphate isomerase (MPI) of *Haemonchus contortus* males for isolates and laboratory populations.

		Ab	Bf	Cp	Pb	St	As	Sy
Isolates	**Ab**	0						
	Bf	0.06*	0					
	Cp	0.04	0	0				
	Pb	0	0.04	0.01	0			
	St	0	0.06*	0.03	0	0		
Laboratory populations	**As**	0.08*	0	0	0.05	0.07*	0	
	Sy	0.01	0.01	0	0.01	0.01	0.02	0

* significant F_{st} at $P < 0.05$.

Table V. – Genetic differentiation (F_{st}: non random mating between populations) between *Haemonchus contortus* isolates and laboratory populations.

consensus tree constructed on majority rule (Fig. 1) indicated that most isolates or assemblage populations were different (their relative position was never modified along the 100 resamplings: 100 % bootstrap value). The synthetic strain and isolate Pb had poor bootstrap values (the same tree was found in 76 and 74 % of resamplings) indicating that their differences with other isolates/laboratory populations were not obvious. The intermediate position of Sy in the tree and lower bootstrap values showed that it was "intermediate" within the all set of studied populations: it resembled equally all isolates.

DISCUSSION

ISOLATES OF *HAEMONCHUS* SP. WERE *H. CONTORTUS*

The identification based on spicule morphometrics did show that all studied specimens were *H. contortus*. This was not expected as other species have been recorded in sheep or goats in Mauritania (Jacquiet *et al.*, 1998) or sheep in Brazil (Amarante *et al.*, 1997), when small ruminants are grazed

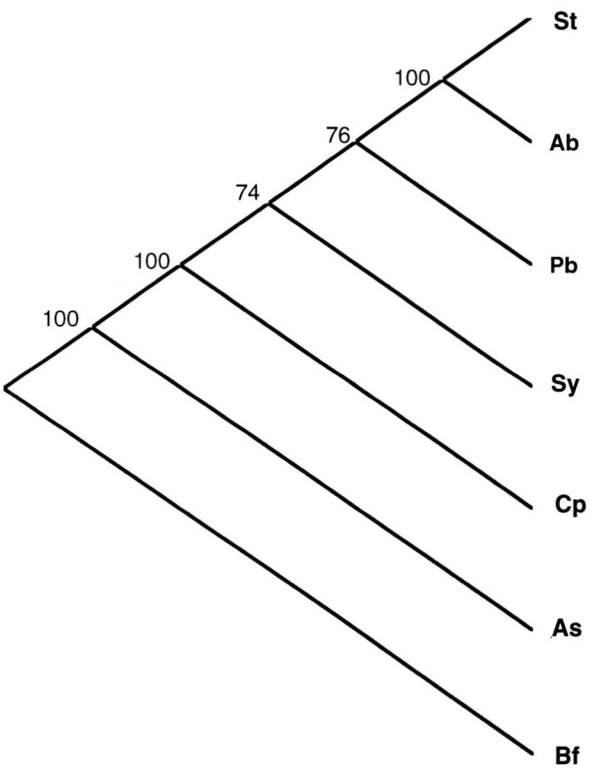

Fig. 1. – *Haemonchus contortus* isolates (Ab, Bf, Cp, Pb, and St) and laboratory populations (As and Sy): consensus tree established by neighbour-joining and majority-rule (based on 100 bootstraps) of Reynolds distance based on allozyme study (Malate dehydrogenase and Mannose phosphate isomerase). The figures represent the percentage of resamplings that indicates the same position in the tree for isolates or laboratory populations.

with cattle. The vulvar morph proportions of females did not correspond to the ecotype *H. contortus* utkalensis described by Das & Whitlock (1960), which was the ecotype recovered in humid tropical areas. No other ecotype previously described matched with the obtained proportions and this is possibly due to local adaptation. A large deficiency in heterozygotes at the MPI locus was found in several isolates (Ab, Bf and Pb) and posed question. The explanatory hypotheses (Borsa *et al.*, 1991; Gasnier & Cabaret, 1998) which had been frequently suggested to explain deficiency in heterozygotes are: 1) the misscoring of null heterozygotes as homozygotes, 2) the selection against heterozygotes before recruitment in the sample, 3) an assortative fertilisation, 4) the Walhund effect (e.g. assemblage of two populations with very different allelic proportions) and finally 5) the existence of cryptic species or semi-species (as seen in another trichostrongyle: Gasnier & Cabaret, 1996). The misscoring of null heterozygotes, selection against heterozygotes and assortative fertilisation are not probable as heterozygote deficiency was recorded in some isolates and not in others. The Wahlund effect could be suspected in some isolates if we assume that breeders exchange infected hosts and that isolates have very different allelic frequencies. The synthetic strain should conversely have Hardy-Weinberg equilibrium, which was not the case. The existence of cryptic species or semi-species cannot be ruled out but needs further investigations.

Isolates of *H. contortus* were slightly different

Isolates were different based on morphometrics of males, proportions of female morphs and allozyme frequencies. The Les Saintes isolate had the male largest spicule length and female worm length which resulted in higher fecundity. The large size of worms in Les Saintes remains unexplained. The proportions of the three female morphs in Les Saintes were equivalent, which was not the case in other isolates, and it was significantly different from Pb isolate. Differences in proportions of female morphs have been recorded by Mc Kenna (1971) on a limited geographic scale in New-Zealand, as in our present results, the rule being the similarity of proportions in large areas at various periods of the year (Le Jambre, 1968). The Saintes isolate was different from Bf but much alike to others on the basis of F_{st} in allozyme study and the constructed tree showed two groups: St and Ab *versus* Pb, Cp and Bf. The Les Saintes isolate was different from all other isolates whatever method we used. This could be possibly explained by a founder effect (few worms introduced into this small island and submitted to dry climatic conditions) which induced random genetic drift. The fact that the other isolates were globally alike could be due to the fact that climatic environment in all sites was very effective for the development of *H. contortus* (Kates,1950) and that breeders exchanged infected animals, which reduced the possibility of divergence between populations. The normally low values of F_{st} should not be misleading: the *H. contortus* isolates from Guadeloupe were as different as those found in Portugal, Azores and Cabo Verde (Neto-Padre, personnal communication, 1999), which represents a large array of climatic conditions.

Synthetic strain was representative of all isolates

The establishment of a synthetic strain is difficult for two reasons: will it be representative of the isolates it comes from, and will it evolve during its construction? The long-time laboratory reared trichostrongyles nematodes undergo changes in their life-traits (Chehresa *et al.*, 1997) or in their genetic characteristics (Gasnier *et al.*, 1992; Gasnier & Cabaret 1998). During the few laboratory passages in our experiment, we noted a decrease of female knobbed vulvar morph. Das & Whitlock (1960) found that goats harboured more knobbed females than sheep, and this could explain why we obtained a lower percentage of knobbed females during acclimation in the laboratory of isolates from goat to lambs. We also noticed a strong decrease in fecundity during successive infections in laboratory conditions. This might be due to the change from the unstable environment (natural conditions) into a stable one (laboratory conditions for free living stages, monospecific infection and infection of susceptible lambs) as shown for another nematode, *Teladorsagia circumcincta* (Gasnier & Cabaret, 1998). This might be explained by the accumulation of pseudo-deleterious mutations in a favourable environment whereas the individuals harbouring these mutations would be eliminated in a harsher environment. Another factor could play a role in the decreased size of nematode in the synthetic strain, e.g. the repeated infections of lambs that induced host resistance (2 % establishment rate *versus* 20 % in naive lambs: data not shown). It has been shown that resistance correlates with small size of female *Teladorsagia circumcincta* (Stear *et al.*, 1999), another trichostrongyle. Curiously, lambs, although submitted to long-acting corticosteroids, were not sufficiently immunosuppressed and they apparently mounted resistance to infection.

The allozymes are usually neutral (e.g. they are not selected by any kind of infection regimen: Gasnier *et al.*, 1996) and consequently any departure from neutrality should be seriously investigated. The assemblage was not really a good reflect of the isolates whereas the synthetic strain remained in the mid-range of allo-

zyme variability. In that respect, the synthetic strain was really synthetic. It can be concluded that assemblages are neither in life-traits nor allozymes a good representative of isolates whereas the generation issued from the first assemblage (the synthetic strain) is a better representative of the isolates. These experiments show that a synthetic strain is readily available, and then investigators could obtain strains which are as variable as a group of isolates. These synthetic strains are then of real interest if one wants to organise selection of nematode on whatever ecological traits. The remaining question is: for how long will the synthetic remain representative of the isolates in very favourable environment like the one offered in experimental conditions? The decrease in fitness observed in Trichostrongylids during three (Gasnier *et al.*, 1992; Gasnier & Cabaret, 1998) or 11 generations (Chehresa *et al.*, 1997) of acclimation can be compared to that obtained in *Drosophila melanogaster* (Shabalina, 1997) maintained under relaxed natural selection during 30 generations (somewhat similar to trichostrongyle nematode maintenance in laboratory). The evolution of nematodes under laboratory conditions is a source of questions on experimental models in helminthology.

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