Fitness of benzimidazole-resistant and -susceptible worms of *Teladorsagia circumcincta*, a nematode parasite of small ruminants

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(*Received 20 April 1998; revised 20 June 1998; accepted 20 June 1998*)

SUMMARY

We compared, some fitness-related traits of benzimidazole resistant (*rr*) and susceptible (*rS*, *SS*) worms of *Teladorsagia circumcincta*, a gastrointestinal parasite of the small ruminants, under laboratory conditions. PCR was used to determine the genotypes (*rr*, *SS*, *rS*) and the fitness of each was compared within the same strain. There was no significant difference in egg production, development rate from egg to infective larvae stage, establishment of these larvae in the host or the survival of adult worms and infective larvae for the 3 genotypes. The same results were obtained for the establishment rate of larvae in the host and the production of infective larvae under conditions of strong competition between resistant and susceptible worms. The fact that there were no differences in fitness suggests that the installation of benzimidazole resistance in a worm population is irreversible. This agrees with field observations.

Key words: ruminant parasite, *Teladorsagia circumcincta*, benzimidazole resistance, fitness, PCR genotyping.

INTRODUCTION

Teladorsagia circumcincta is one of the most common gastrointestinal parasites of sheep and goats in temperate zones. The economic losses caused by these parasites have led farmers of small ruminants to use anthelmintics intensively. Benzimidazoles (BZ) derivatives have become the most widely used of these drugs but resistance to them is now common throughout the world (Bjorn, 1994, Conder & Campbell, 1995). The genetics of this resistance to BZ is linked to changes in isotype 1 β-tubulin that is the target of BZ. The replacement of the amino acid phenylalanine at position 200 by a tyrosine seems to be the main change producing resistance (Kwa, Veenstra & Roos, 1994; Kwa *et al*. 1995; Elard, Comes & Humbert, 1996; Elard & Humbert, unpublished observations).

The rate at which resistance develops depends on the proportion of the population subjected to selection and the relative fitness of the genotypes carrying the gene conferring resistance. In our model, only adult worms in the hosts were treated with BZ anthelmintic but most of the parasites are larvae free on pastures. These larvae constitute a reservoir of BZ-susceptible genotypes during the first step of the resistance selection. The fitness of susceptible heterozygous (Phe/Tyr) and homozygous (Phe/Phe) individuals of *T. circumcincta* is close to zero under selection pressure by BZ treatment, since only homozygous (Tyr/Tyr) individuals survived anthelmintic treatment (Elard & Humbert, unpublished observations). However, we do not know the relative fitness of resistant or susceptible genotypes without BZ treatment. This question is still very important because a selective disadvantage of the *rr* genotype without BZ treatment can be useful for managing resistance to BZ. We also need to know the fitness of heterozygous individuals (*rS*) relative to homozygous (*SS*), because the resistance gene is carried by these *rS* individuals during the early stages of resistance development, when few *rr* individuals are present (Roush & McKenzie, 1987).

Several studies on other Trichostrongyle species have tried to compare the fitness of resistant or susceptible strains, but the results have been contradictory. Kelly *et al*. (1978) found that BZ-resistant strains of *Haemonchus contortus* were more infective and fecund than unresistant strains, while Maingi, Scott & Pritchard (1990) obtained opposite results for other strains of the same species. But the origins (and probably the life traits) of the resistant and susceptible strains compared were different, which could introduce bias into this kind of study.

It would be better to compare the fitness of BZ-resistant and susceptible worms from the same strains. We have developed a molecular tool (Humbert & Elard, 1997) for determining the genotype (*rr*, *rS* or *SS*) of *T. circumcincta* for this

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https://doi.org/10.1017/S0031182098003436 Published online by Cambridge University Press

purpose. We have compared the fitness of BZ-resistant and susceptible parasites under different conditions of competition. We looked for fitness disadvantages that result from resistance by 2 methods. In one, we measured components of fitness, such as egg production, larval development, establishment rate of the larvae in the host and survival of the adult worms and infective larvae L3 for each genotype. In the second, we monitored genotypic frequencies for 3 generations of parasites that were not submitted to anthelmintic benzimidazole pressure.

MATERIALS AND METHODS

Parasites

T. circumcincta is a trichostrongyle parasite which has a direct cycle. Eggs laid by the females, develop into 3rd-stage larvae, the infective stage L3, on pasture (Fig. 1). Two strains just isolated from the field for these experiments, were passaged once on sheep under laboratory conditions: a susceptible strain (SuBOU; 100% of *SS* genotype in the adult worm population; 100% faecal egg count reduction after BZ treatment) and a resistant strain (ReGP; $rr = 30\%$, $SS = 19\%$, $rS = 51\%$ in the adult worm population) in which the BZ resistance was estimated by the method of Coles *et al.* (1992) (ReGP, $LD_{50} = 0{\cdot}15$ μg/ml).

Genotyping of the worms

A phenylalanine (TTC) or a tyrosine (TAC) at residue 200 of the β-tubulin was detected as described by Humbert & Elard (1997) and Elard, Cabaret & Humbert (1999). PCR was used to amplify allele-specific products (TTC or TAC) using 4 primers, 2 allele-nonspecific primers and 2 allele-specific primers. Three fragments can be obtained (Fig. 2).

For the genotyping of the larvae, these larvae were exsheathed by incubation in 0·16% sodium hypochlorite for 30 min. Each larvae was then placed in a 200 μl PCR tube with 2 μl of sodium hypochlorite and freeze-thawed 3 times. They were then incubated overnight in digestion solution (3 μl of Tris–EDTA buffer, 2 μl of proteinase K and 2 μl of Tween 20) and at 95 °C for 1 h. Seven μl of this solution were taken for the PCR which allows the genotyping (Phe or Tyr) of the parasites.

Egg production and larval development

The egg production and larval development of BZ-resistant and susceptible worms (Fig. 1A) were compared using females recovered from the abomasum of 2 slaughtered sheep infected with the ReGP population. Each female was placed in a well of a 24-well culture plate containing 1 ml of agar (0·8 g Agar (Sigma) in 40 ml of bactericin-free water). Each well also contained 300 μl of medium (750 μl of penicillin (5000 IU/ml)–streptomycin (5000 UG/ml) and 1·25 ml nystatin (1000 IU/ml) in 100 ml of NCTC-135 supplemented with L-glutamine (all from Gibco-BRL)). The number of eggs produced by each female after 5 h of incubation at 37 °C were counted. The females were then removed from the well and typed to determine their genotype (*rr*, *SS*, *rS*). The plates were placed at 23 °C and the number of larvae (L1) in each well at 48 h was estimated.

Establishment of the infective stage (larvae 3) in the host

The establishment of the genotypes (*rr*, *SS*, *rS*) in the host (Fig. 1B) was compared by infecting male lambs orally with infective larvae (L3) in which the proportion of each genotype was known. In the first experiment, 6 lambs were infected with 4000 ReGP larvae. The lambs were killed on day 35 or on day 60. At necropsy, adult worms were counted and the proportions of each genotype in each adult worm population were estimated. In the second experiment, 6 lambs were infected with 2000 SuBOU larvae and 2000 ReGP larvae while in the third experiment, 6 lambs were infected with 7000 SuBOU larvae and 7000 ReGP larvae.

From adult to infective-stage larvae

We compared the proportions of each genotype in the adult worm populations and in the infective larvae obtained from these populations (Fig. 1C). Samples of faeces were collected from each lamb from day 25 in the above experiments. These faeces were cultured to obtain infective-stage larvae (L3) (Rossanigo & Gruner, 1995). The proportion of each genotype in these infective larvae was estimated for each lamb.

Comparison of the genotype frequencies after 3 generations

We compared the genotype frequencies of adult worm populations after 3 generations maintained in laboratory conditions without BZ treatment. Parasite strains were passaged in lambs and larvae (L3) recovered from faecal cultures were used to infect the next host. One experiment was performed with the ReGP strain (4000 larvae) and the other with a mixture from the ReGP and the SuBOU strains (7000 larvae of each strain). Adult parasites were recovered from the abomasum of the lambs after the 3 passages.

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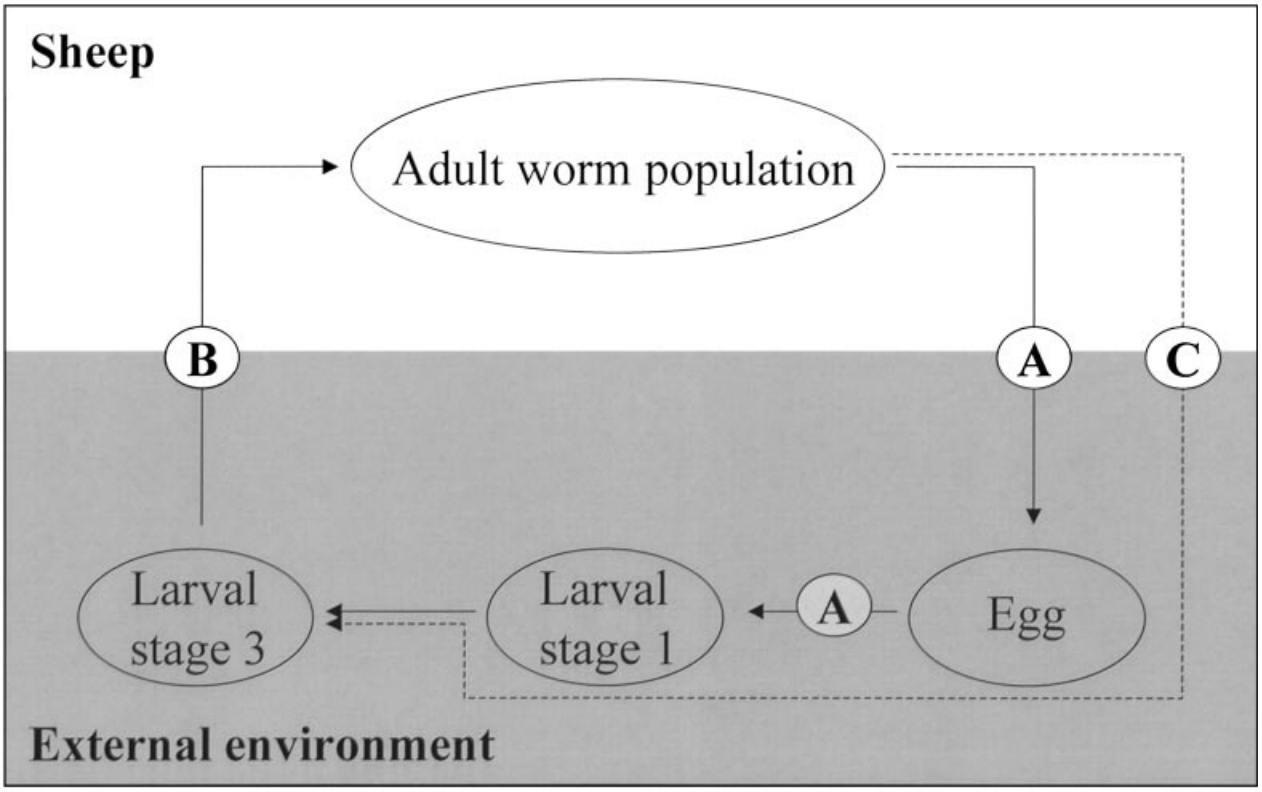

Fig. 1. Life-cycle of *Teladorsagia circumcincta* and the fitness related traits studied. (A) Egg-laying and development from egg to 1st-stage larvae. (B) Establishment of the infective larvae L3 in the host. (C) Production of 3rd-stage larvae by an adult worm population.

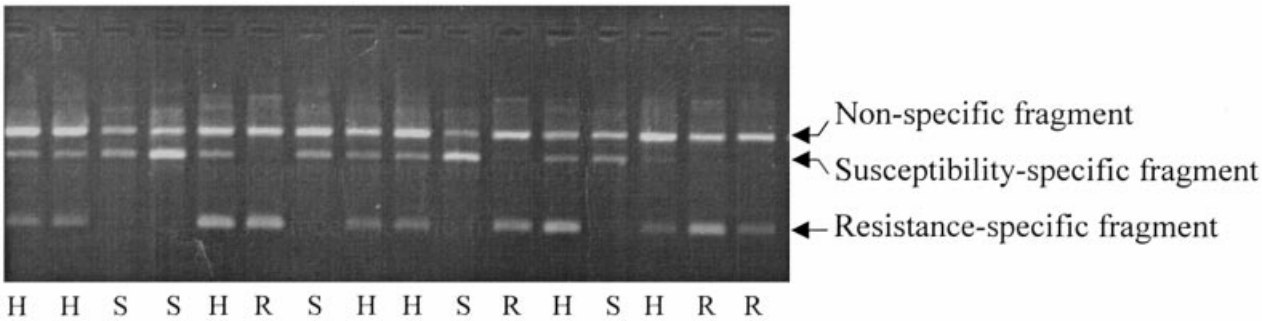

Fig. 2. PCR genotyping of adult worms from the resistant population ReGP. The first amplified fragment was allele non-specific and the 2 other were allele-specific. H = heterozygous *rS*; R = homozygous *rr*; S = homozygous *SS*.

RESULTS

Egg production and development from the egg to the 1st-stage larvae

Females were recovered from 2 lambs infected with 4000 strain ReGP larvae. The females ($n = 72$) isolated from lamb 1 laid 52 eggs ($\sigma = 26$) while the females isolated from lamb 2 ($n = 72$) laid 63 ($\sigma = 24$). The difference between these 2 means was not significant (Student's *t*-test, $P < 0{\cdot}05$). The distribution of females as a function of the number of eggs laid is shown Fig. 3. This distribution fits the normal distribution curve ($\chi^2 = 15{\cdot}5$, degree of freedom $\nu = 8$, $P < 0{\cdot}05$).

In the first lamb, 61 females were genotyped and the proportions of females, that laid more or less than 50 eggs (close to the average value), were determined at each genotype (Table 1). There was no significant difference between the 3 genotypes ($\chi^2 = 0{\cdot}02$, $\nu = 2$, $P < 0{\cdot}05$).

The numbers of 1st-stage larvae obtained from the eggs in lambs 1 and 2 were significantly different. Only 12 % of the eggs from lamb 1 developed to 1st-stage larvae, while 24 % of the eggs from lamb 2 did so. There was no significant difference ($\chi^2 = 0{\cdot}23$, $\nu = 2$, $P < 0{\cdot}05$) in the rates of development from egg to the 1st-stage larvae for the 3 genotypes in lamb 1 (Table 1).

Establishment of the infective-stage larvae in the host

An average of 42 % of larvae became established in the host (Table 2), but significantly fewer larvae

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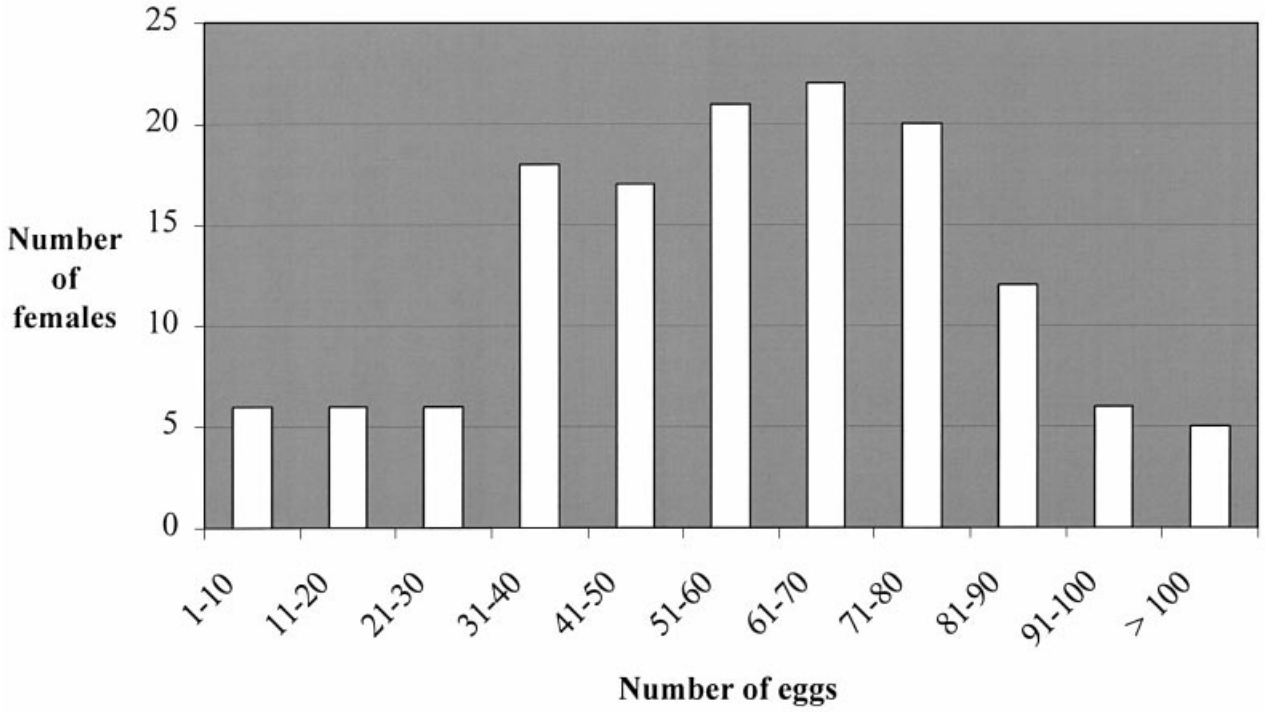

Fig. 3. Egg outputs of female resistant worms of the ReGP population. This distribution fits a normal distribution (χ^2 test, $P < 0{\cdot}05$).

Table 1. Proportions of each genotype in females laying more or less than 50 eggs and in the developmental rates from eggs to 1st-stage larvae

	Genotype		
	rS	*rr*	*SS*
Egg-laying < 50	16 (50%)	9 (28%)	7 (22%)
Egg laying ⩾ = 50	15 (52%)	8 (27%)	6 (21%)
Development rate from egg to L1 < 10%	18 (51%)	9 (26%)	8 (23%)
Development rate from egg to L1 > = 10%	13 (50%)	8 (31%)	5 (19%)

were established in Experiment 3 (14000 larvae ingested/lamb) than in Experiment 2 (4000 larvae ingested/lamb) (Mann–Whitney *U*-test, $P < 0{\cdot}05$).

Only larvae from the ReGP population were given to lambs in Exp. 1 (Table 2). The proportions of each genotype (*rr*, *SS*, *rS*) in the 6 adult worm populations (lamb 2.1 and lamb 2.6) were essentially the same (χ^2 test, $P < 0{\cdot}05$) except for the worm population in the lamb 2.5 which differed from those of lambs 2.2 ($\chi^2 = 7{\cdot}5$, $\nu = 2$) and 2.3 ($\chi^2 = 6{\cdot}5$, $\nu = 2$). The proportions of each genotype in the larvae population used to infect the lambs, and in the resulting adult worm populations were the same, except for lamb 2.2 ($\chi^2 = 10{\cdot}1$, $\nu = 2$).

In the second experiment (Table 2), the lambs were infected with 2000 ReGP larvae and 2000 SuBOU larvae. The proportions of each genotype in the 6 adult worm populations (lamb 2.11 to lamb 2.16) were the same except for population 2.13, which differed significantly from populations 2.11 ($\chi^2 = 15{\cdot}5$, $\nu = 2$), 2.12 ($\chi^2 = 7{\cdot}3$, $\nu = 2$), 2.15 ($\chi^2 = 19{\cdot}6$, $\nu = 2$) and 2.16 ($\chi^2 = 8{\cdot}7$, $\nu = 2$). The proportions of each genotype in the larvae given to lambs and in the resulting adult worm populations in these lambs were not significantly different, except for population 2.13 ($\chi^2 = 19{\cdot}7$, $\nu = 2$).

In the third experiment, the lambs were infected with 7000 ReGP larvae and 7000 SuBOU larvae. Again, there was no significant difference in the proportions of each genotype in the 6 adult worm populations (lamb 2.21 and lamb 2.26), and there was no difference in the proportions of each genotype in the larvae given to lambs and in the resulting adult worm populations.

From adult to infective-stage L3

There was no difference (χ^2 test, $P < 0{\cdot}05$) between

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Table 2. Genotypes (*rr*, *SS*, *rS*) in adult worm populations after infecting lambs by 3 protocols, and in the larvae derived from these adult worms

Lamb	Date of necropsy p.i.	% of establishment	No. of adults			No. of larvae		
			rr	*rS*	*SS*	*rr*	*rS*	*SS*
Exp. 1: 4000 L3 of the resistant strain ReGP/lamb. The proportions of each genotype in the larvae used to infect the lambs were *rr* = 31, *rS* = 48, *SS* = 32								
2.1	D35	64	30	36	13	10	17	12
2.2	D35	57	49	49	17	6	22	11
2.3	D35	10	15	20	4	12	19	8
2.4	D60	25	12	20	7	10	15	3
2.5	D60	55	10	15	13	—	—	—
2.6	D60	43	12	15	11	10	16	10
Ext. 2: 2000 L3 ReGP + 2000 L3 SuBOU/lamb. The proportions of each genotype in the larvae used to infect the lambs were *rr* = 16, *rS* = 24, *SS* = 71								
2.11	D52	45	3	8	26	2	16	19
2.12	D60	66	7	9	21	10	17	13
2.13	D35	52	17	34	23	1	10	19
2.14	D35	18	9	10	19	—	—	—
2.15	D60	53	8	6	33	4	12	23
2.16	D28	67	8	8	22	5	14	19
Exp. 3: 7000 L3 ReGP + 7000 L3 SuBOU/lamb. The proportions of each genotype in the larvae used to infect the lambs were *rr* = 16, *rS* = 24, *SS* = 71								
2.21	D35	29	17	12	49	11	15	23
2.22	D60	17	21	23	49	5	25	45
2.23	D60	24	12	16	35	11	28	39
2.24	D35	42	16	21	42	5	23	52
2.25	D60	40	6	7	19	10	11	18
2.26	D35	41	4	3	12	4	5	29

the proportions of each genotype in adult worms and in the infective L3 larvae derived from these adults, except in lamb 2.2 (Exp. 1; $\chi^2 = 10{\cdot}1$, $\nu = 2$), 2.13 (Exp. 2; $\chi^2 = 11{\cdot}1$, $\nu = 2$) and 2.22 (Exp. 3; $\chi^2 = 8{\cdot}3$, $\nu = 2$) and 2·24 (Exp. 3; $\chi^2 = 6{\cdot}9$, $\nu = 2$).

Finally, we looked for differences between the larvae used to infect the lambs and the larvae obtained from worm populations which established in these lambs. We found no significant difference (χ^2 test, $P < 0{\cdot}05$) in the proportions of each genotype except in lambs 2.11 ($\chi^2 = 7{\cdot}4$, $\nu = 2$) and 2.12 ($\chi^2 = 11{\cdot}9$, $\nu = 2$).

Survival of adult worms and infective larvae L3

The lambs were killed at 35 or 60 days post-infection (Table 2). As previously related in the establishment analysis of the infective-stage larvae in the host, there was generally no significant difference in the proportions of each genotype in adult worm populations. So, no decrease of the proportion of the *rr* genotype was observed in the worm populations of lambs killed 60 days post-infection in comparison with worm populations of lambs killed 35 days p.i.

The same type of result was obtained for the infective-stage L3 larvae. There was no significant difference (χ^2 test, $\nu = 2$; $P < 0{\cdot}005$) when ReGP larvae were compared at 5 month intervals after conservation at 8 °C (First estimation: *rr* = 15, *rS* = 10, *SS* = 23; Second estimation 5 months later: *rr* = 15, *rS* = 16, *SS* = 20; $\chi^2 = 1{\cdot}5$) and when larvae resulting from 3 lambs of the third experiment, were compared at 3 month intervals after conservation at 8 °C (*Lamb* 1, First genotyping (G1): *rr* = 2, *rS* = 14, *SS* = 21; Genotyping 3 months later (G3): *rr* = 3, *rS* = 11, *SS* = 24, $\chi^2 = 0{\cdot}75$ – *Lamb* 2, G1: *rr* = 3, *rS* = 12, *SS* = 23; G3: *rr* = 8, *rS* = 16, *SS* = 16, $\chi^2 = 4{\cdot}05$ – *Lamb* 3, G1: *rr* = 3, *rS* = 10, *SS* = 27; G3: *rr* = 2, *rS* = 13, *SS* = 25, $\chi^2 = 0{\cdot}67$).

Comparison of the genotype frequencies after 3 generations

The frequencies of each genotype in the ReGP population were compared after 3 generations produced under laboratory conditions. There was no significant difference ($\chi^2 = 0{\cdot}10$, $\nu = 2$, $P < 0{\cdot}05$) in the proportions of each genotype in the larvae used at the beginning of this experiment (*rr*: 31; *SS*: 32; *rS*: 48) and the proportion of each genotype after 3 generations (*rr*: 25; *SS*: 28; *rS*: 43). The same type of result (no significant difference; $\chi^2 = 1{\cdot}95$, $\nu = 2$, $P < 0{\cdot}05$) was obtained after 3 generations of lambs infested with a mixture of 7000 SuBOU larvae and 7000 ReGP larvae. The proportion of each genotype in larvae was *rr* = 16, *SS* = 71, *rS* = 24, and in the

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third generation of adult worms: $rr = 20$, $SS = 58$, $rS = 28$.

DISCUSSION

We have looked for fitness disadvantages that result from the acquisition of the benzimidazole resistance by *T. circumcincta*, a nematode parasite of small ruminants. Those disadvantages were suspected because the β-tubulin gene is highly conserved. The peptide sequences of β-tubulin from 2 nematodes species belonging to different trichostrongyle genus (*T. circumcincta* and *Haemonchus contortus*) were very similar (homology > 99%; Elard *et al*. 1996). Partial sequencing of the β-tubulin gene in more than 100 individuals of *T. circumcincta* showed considerable polymorphism in the non-coding sequences and numerous synonymous mutations, but no amino-acid substitution (Elard & Humbert, unpublished observations). These results suggest that non-synonymous mutations on the β-tubulin gene are probably strongly counter-selected. As the benzimidazole resistance is linked to the replacement of a phenylalanine at residue 200 by a tyrosine, a decrease in fitness was also suspected in resistant genotype.

There does not appear to be any significant difference in the fitness-related traits of the 3 genotypes (*rr*, *SS*, *rS*). The unimodal distribution (instead a polymodal distribution) of the eggs laid by the females from a population containing 3 genotypes (ReGP) seems to indicate that there is no difference in the egg-laying of females at each genotype. The absence of difference between genotypes in the proportion of females which laid fewer or more than 50 eggs confirms this.

We also obtained no evidence for a difference in the establishment of the infective larvae in the host between the 3 genotypes. The proportions of each genotype in the larvae used to infest the host and those in adult worm populations were the same in the 3 experiments except for 2 adult populations. One (lamb 2.2) had a relative excess of resistant homozygous adults, while the other (lamb 2.13) had an excess of heterozygous adults. These 2 exceptions were probably due to sampling artifacts, because the larvae used to infect the lambs and the larvae obtained from adult worms installed in these lambs had the same proportions of each genotype. The fact that the same results were obtained in all 3 experiments is very interesting, because they tested different conditions of competition. The frequencies of resistant and susceptible alleles were the same (50/50) in the first and there was little competition between the larvae for establishment in the host. But in the second and third experiments, the resistant allele was in a minority (25%), and competition between the larvae for establishment in the host was great in the third experiment, as over 14000 larvae were given to the lambs and only 2000–6000 adult worms were recovered. Despite these conditions, the resistant genotype (*rr*) showed no fitness disadvantage for the establishment in the host.

There were significant differences in the proportions of each genotype in the adult worm populations and in the larvae derived from them in 4/18 cases. This difference was not linked to the proportion of each genotype in larvae populations in lamb 2.2, but to the proportion of adult worms having an 'excess' of resistant homozygous worms. The proportions of each genotype in this larvae population were similar to those in the larvae populations in the other lambs in this first experiment. The differences in lamb 2.13 may be similarly explained. But, this explanation cannot be valid for lambs 2.22 and 2.24. The larvae from these 2 lambs had a smaller fraction of resistant homozygous genotypes (*rr*), while the frequencies of the resistant allele (*r*) in these larvae populations were unaltered (23% for the lamb 2.22, and 21% for the lamb 2.24).

The few significant differences found among all the experimental results might relate to statistical bias. The existence of Type I error (rejecting H_0 when H_0 is correct, H_0 being the absence of difference) increases when multiple tests are carried out. At a 5% level of risk, 5 mistakes are expected when 100 tests are performed. In this work, more than 100 χ^2 tests were performed and false significant results might occur.

All these results suggest that the replacement of a phenylalanine at residue 200 of the β-tubulin gene linked to BZ resistance probably has no major effect on the fitness of the mutant individuals. This finding was obtained with different competitive conditions under which fitness was assayed. This is important because several studies have shown that mutational pressure on fitness depends on these competitive conditions (see for example Shabalina, Yampolsky & Kondrashov, 1997). However, our study was only conducted under laboratory conditions and suffers from the problem of uncertain relevance to field conditions (Taylor & Feyereisen, 1996). For this reason, we develop actually several protocols in the field with the ReGP strain to compare the fitness of each genotype. Another problem is that small fitness differences between genotypes cannot be detected due to insufficient sample sizes. This should not be a major problem as modelling studies (data not shown) reveal that only large fitness differences (> 20%) between genotypes have a significant impact on the allelic frequencies in less than 20 generations (5–10 years in the field).

There are several lines of support for the absence of major effects of this mutation on the fitness of the worms. The first is that other mutations linked to BZ resistance have been described in the β-tubulin gene of *Caenorhabditis elegans* and in several species of

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fungi (Elard *et al*. 1996). The β-tubulin gene of *Aspergillus nidulans* has 7 mutational sites linked to BZ resistance but 3 of them (codons 50, 134, 257) are temperature sensitive. Strains with these mutations are likely to have a reduced fitness (Koenraadt, Somerville & Jones, 1992). But the only change reported in trichostrongyle species is the substitution of a Tyr for Phe at residue 200. This is probably because this mutation has less effect on the fitness of the worms than other mutations that can confer resistance to BZ. The second element is that a tyrosine is at the residue 200 of the β-tubulin in many organisms (all vertebrate sequences). This residue lies in the strand B6 which leads to the intermediate domain (Nogales, Wolf & Downing, 1998). This domain is highly conserved in vertebrates, invertebrates and fungi, and residue 200 is the sole polymorphic site in this region. The fact that there may be a tyrosine or a phenylalanine at residue 200 of this conserved region suggests that this substitution has no great influence on the fitness of the organisms. Lastly, several studies have found no reversion of the BZ resistance in worm populations not treated with BZ (Dash, 1986; Hall, Ritchie & Kelly, 1982; Martin *et al*. 1988), or given another anthelmintic (Borgsteede & Duyn, 1989). Similarly, genotyping of the adult worm population ReGP in 1991 and 1996 (data not shown) revealed no difference in the proportions of each genotype (*rr*, *SS*, *rS*). Yet, in this time, the worms have encountered very slight BZ selection pressure because another anthelmintic (Levamisole) was used for 2 years and only 2 BZ treatments were given in all during the 3 other years (data not shown).

In conclusion, we find strong evidence, that the mutation on the β-tubulin gene involved in the BZ resistance has not a major effect on the fitness of the resistant mutant worms (*rr*) of the *T. circumcincta* species under laboratory conditions. These results agree with field observations showing that BZ resistance seems to be irreversible in a worm population. This finding has great consequences for the management of the BZ resistance in farms. For example, several studies have suggested rotating the use of anthelmintics from different groups (Pritchard *et al*. 1980; Dash, Newman & Hall, 1985; Coles & Roush, 1992), but such a strategy has no effect on resistant worms if their fitness is similar to that of susceptible worms.

L. Elard was supported by a grant from the Région entre and the INRA. We thank J. Cabaret for stimulating discussions and the anonymous reviewers for their useful commentaries. The English text was checked by Dr Owen Parkes.

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