Serial passage in resistant sheep drives the infectivity and fitness of Teladorsagia circumcincta in susceptible lambs: Experimental evidence

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Serial passage in resistant sheep drives the infectivity and fitness of *Teladorsagia circumcincta* in susceptible lambs: Experimental evidence

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Abstract

Gastrointestinal nematodes (GIN) of small ruminants have adapted their life history strategies to thrive in diverse and fluctuating environments. Environments which alter their expression of life traits may also drive changes in the infection or transmission dynamics, particularly if transferred to a foreign setting. This study aimed to explore how repeated exposure to a resistant sheep host environment would alter the life history traits and infection dynamics of *Teladorsagia circumcincta* when consequently infected in susceptible lambs. Following just three generations of passage in resistant sheep, *T. circumcincta* significantly increased their infectivity and fitness in susceptible lambs compared to a control population. This is the first evidence to indicate the resistant host environment can drive such rapid changes in the expression of GIN life traits, with potentially undesirable epidemiological outcomes.

Keywords

Gastrointestinal nematodes, resistant sheep, life traits, infection dynamics, infectivity, fitness

1. Introduction

Gastrointestinal nematodes (GIN) are highly successful parasites of small ruminants. Despite numerous attempts at control, they maintain a global distribution at often high infection intensities. Capable of adapting to diverse and fluctuating environments, GIN have evolved dynamic life histories (i.e. patterns of growth, reproduction, and survival), which can be modified to optimise their fitness relative to a specific environment [1, 2]. The adaptation of GIN life history traits to local conditions has been documented in several studies. For example, in the deserts of Mauritania, female *Haemonchus contortus* synchronise their egg production to coincide with the brief rainy season, where moisture is essential to the survival and development of the free-living stages [3, 4]. Differences in GIN life traits have also been recorded across a more discrete, microgeographic scale. A study comparing five populations of *Teladorsagia circumcincta* obtained from farms within a 60 km^2 radius in France observed distinct developmental schedules of the free-living stages, specialised to facilitate transmission within the local microclimate and husbandry conditions [5].

Environments which alter the expression of GIN life traits may also drive changes in the infection dynamics, particularly if transferred to a foreign setting. In a comparative study of two *H. contortus* isolates, an allopatric isolate obtained from the GIN resistant Blackbelly breed of sheep in Guadeloupe (French West Indies), and a sympatric isolate obtained from the GIN susceptible Romane breed of sheep in France, found the foreign Guadeloupe isolate to be significantly more infective in French Romane lambs, with almost triple the establishment capacity compared to the local French isolate [6]. The environments from which these isolates were obtained likely varied in a myriad of ways (i.e. climate, husbandry, etc.), each of which may have had a bearing on their life histories. Disentangling whether a specific environmental factor, such as resistant sheep, can have unfavourable evolutionary consequences on the parasite's infection dynamics, is crucial to the development of sustainable control strategies.

Depending on the protective mechanism(s), resistant sheep can limit GIN success by preventing the establishment of incoming larvae, suppressing the growth and fertility of adult worms, and/or by expelling the worms [7]. The moderate heritability of the GIN resistance trait has resulted in its increasing inclusion into selective breeding programs, as an inexpensive means to improve flock health and productivity [8, 9]. Irrespective of their genetic backgrounds, however, young lambs remain susceptible to GIN infection until their protective immune capability is fully developed, which may take up to 10 – 12 months depending on exposure to infection [10, 11]. The aim of this study was to

evaluate how repeated passage in resistant sheep may impact the life traits and infection dynamics of the GIN *T. circumcincta* when consequently infected in susceptible lambs. The results highlight the rapidity with which changes to GIN life traits, and infectivity, can take place, with potentially adverse consequences on disease epidemiology and control.

2. Materials and Methods

2.1 Ethical Committee

All the experiments recorded in this protocol were accepted by the Ethical Committee Val de Loire (no19) under the no. 2012-08-10.

2.2 Teladorsagia circumcincta Isolates: Tcirc^{R}and Tcirc^{S}

The study created two *T. circumcincta* isolates from a single founding population; one passaged through resistant sheep to produce Tcirc^{R}, the other passaged through susceptible sheep to produce Tcirc^{S}, which served as a control population. The isolates were selected in their respective hosts over three generations.

2.2.1 First Generation (F1)

The first generation of Tcirc^{S} and Tcirc^{R} isolates were obtained from lambs which had been selectively bred over three generations to express either GIN susceptible or resistant phenotypes based on divergent faecal egg counts (FEC) [12]. In brief, 22 female lambs selected for susceptibility and 21 female lambs selected for resistance over three generations were grazed together on *T. circumcincta* contaminated pasture. The *T. circumcincta* was susceptible to benzimidazole (BZ) based on zero FEC post treatment, and on the absence of a tyrosine residue 200 on the β-tubulin gene in adult males [13]. At the end of the grazing season, the two lambs with the highest FEC (e.g. susceptible lambs) and the two lambs with the lowest FEC (e.g. resistant lambs) were sacrificed to obtain the female worms from the abomasum. The *T. circumcincincta* eggs were recovered by opening the ovaries with a scalpel. Eggs obtained from the female worms in susceptible lambs constituted the first generation (F1) of the Tcirc^{S} isolate. Similarly, eggs obtained from the female worms in resistant lambs constituted the first generation of the Tcirc^{R} isolate. The eggs were cultured in lamb faeces under conditions favourable for development into L3 larvae e.g. 23 °C and 70% humidity for 10 days [14]. The L3 larvae were separated from the faeces using the Baermann technique at room temperature over 24 hours [15].

123

124 2.2.2 *Second (F2) and Third (F3) Generation*

125 The Tcirc^{S} and Tcirc^{R} isolates were passaged through susceptible and resistant hosts respectively, a
126 further two times. Given the age, bred and prior experience of GIN infection are known to impact the
127 development and/or strength of sheep protective responses [9], the sheep used in the selective
128 passage of Tcirc^{S} and Tcirc^{R} differed in these respects. The Tcirc^{S} isolate was passaged through two Ile-
129 de-France lambs, a breed considered susceptible to GIN. The lambs were obtained from a zero grazing
130 facility and had no prior experience of GIN infection, as confirmed with zero FEC. Different lambs were
131 used with each passage. The Tcirc^{R} isolate was passaged through two resistant ewes obtained from
132 the selective breeding process mentioned previously [12]. The ewes had previous experience of GIN
133 infection. Prior to each passage, the resistant ewes were treated with pro-benzimidazole netobimin
134 (MSD Animal Health) at 7.5 mg/kg bodyweight (as per the manufacturer's instructions) to clear the
135 previous *T. circumcincta* infection and left for 15 days. The same resistant ewes were used for both
136 passages of Tcirc^{R}.

137

138 The F1 generation of the *T. circumcincta* isolates were passaged through the relevant susceptible
139 lambs (Tcirc^{S}) or resistant ewes (Tcirc^{R}) given at an infective dose of 4000 L3 larvae. The eggs excreted
140 in the sheep faeces were collected and cultured to L3 larvae under the same conditions previously
141 mentioned, to provide the second generation (F2) of isolates. The F2 generation of the *T. circumcincta*
142 isolates were passaged again through the relevant susceptible lambs (Tcirc^{S}) or resistant ewes (Tcirc^{R})
143 given at an infective dose of 4000 L3 larvae. The eggs excreted in the faeces were collected and
144 cultured into L3 larvae as before to provide the third generation (F3) of the *T. circumcincta* isolates,
145 to be compared in the experimental infection in immune-suppressed susceptible lambs (detailed
146 below). The fitness and life history traits of both Tcirc^{S} and Tcirc^{R} were measured throughout the
147 selective passage process (detailed below).

148

149 *2.3 Experimental Study: Infection Dynamics of Tcirc^{S} vs. Tcirc^{R} in Immune-Suppressed Lambs*

150 The experiment infected 10, three-month-old, Ile-de-France male lambs with no prior experience of
151 GIN infection with either the Tcirc^{S} or the Tcirc^{R} isolate. To level any potential variation in the lamb's
152 protective responses, they were administered with long-acting dexamethasone (0.5 mg/kg
153 bodyweight) intramuscularly, given one day prior to infection and eight days after infection. This
154 ensured any potential protective responses were constrained for the entire two-week period in which

T. circumcincta could potential establish [16]. Four of the lambs were infected with the Tcirc^{S} isolate, receiving an infective dose of 2000 L3 larvae. Six of the lambs were infected with the Tcirc^{R} isolate. Due to constraints in the number of Tcirc^{R} L3 larvae recovered from the final F3 passage in resistant ewes, there were not enough Tcirc^{R} to administer the same infective dose. Instead, two of the lambs received 2000 L3 larvae and the remaining four received 1200 L3 larvae.

2.4 Faecal egg counts

Faecal egg counts (FEC) were performed nine times, for F2 and F3 passages, and for the experiment in immune-suppressed lambs. These took place between 21 and 50 days post infection (dpi). A modified McMaster technique [17] was used in a magnesium sulphate flotation solution, accurate to 50 eggs per gram (EPG) of faeces.

2.5 Daily Egg Output based on Quantity of Faecal Matter

To ascertain the daily production of eggs from individual sheep, the total quantity of faecal matter (QFM) produced in a day was calculated based on a formula (back-transformed from logarithm) developed specifically to the conditions of this study. The calculation was based on a linear regression between the logarithm of the weight of faecal excretion collected over a 24 h period and the logarithm of the metabolic weight ($W^{0.75}$) [18, 19] of the Ile-de-France male lambs:

$$QMF = 0.041W^{0.75} \ (P = 0.00;\ r = 0.95).$$

where W is the weight of the individual sheep (kg).

The daily egg output per individual sheep was calculated by multiplying the QFM by the respective EPG determined in the FEC. This was calculated for both *T. circumcincta* isolates for the F2 and F3 passages, and for the experiment in immune-suppressed lambs, at the same time points used for the egg – L3 larvae development ratio (detailed below).

2.6 Egg - L3 larvae development ratio

To measure the ratio of eggs that developed into L3 larvae, 5 x 5g of faecal samples were collected from the sheep between 21 – 50 dpi, for F2 and F3 passages, and for the experiment in immune-suppressed lambs. These were cultured for 10 days under the same climatic conditions outlined previously [14]. The L3 larvae were extracted from the faeces using the Baermann technique over 24 h at room temperature [15] and counted under a stereo microscope (x 50 magnification) to obtain the

number of L3 larvae developed per 5 g of faeces. This was divided by the corresponding FEC, adjusted to the 5 g weight, to provide the ratio of eggs that successfully developed into L3 larvae.

2.7 Establishment of infective larvae

The number of adult worms found in the abomasum at postmortem examination (50 dpi) was counted following the procedure described by [20]. Fourth stage larvae (L4) were also counted, extracted from the mucosa by leaving the abomasum in 37 °C water for four hours. The establishment rate was determined as a percentage of adults and L4 counted from the original infective dose. Establishment was calculated for $Tcirc^S$ following the F2 and F3 passages in susceptible lambs, for $Tcirc^R$ following the F3 passage in resistant ewes, and for both $Tcirc^S$ and $Tcirc^R$ following the experiment in immune-suppressed lambs. It was not possible to sacrifice the resistant ewes to calculate the $Tcirc^R$ establishment rate following the F2 passage as the same animals were required for the F3 passage.

2.8 Fertility

The female fertility was calculated following the methods described in [21]. Fertility, defined as the number of eggs per female per day (EFD), was the average of nine FEC (EPGavg) taken between 21 and 44 dpi, multiplied by QFM excreted per day and divided by the number of females observed at postmortem examination in the abomasum. The following formula was used:

$$EFD = EPGavg \times QFM / Number\ of\ females$$

Fertility was calculated for $Tcirc^S$ following the F2 and F3 passages in susceptible lambs, for $Tcirc^R$ following the F3 passage in resistant ewes, and for both $Tcirc^S$ and $Tcirc^R$ following the experiment in immune-suppressed lambs. It was not possible to calculate the fertility of $Tcirc^R$ following the F2 passage in resistant ewes for the same reason outlined above.

2.9 Absolute (W) *and relative fitness* (w)

Absolute fitness (W) is a function the genotypes' capacity to survive and reproduce (i.e. a culmination of their life traits) based upon the number of reproductive units (i.e. offspring) produced [22]. Owing to the processes of natural selection, genotypes with greater fitness in a given population will produce, on average, more offspring than less fit genotypes. In this case, genotype refers to the isolates $Tcirc^S$ and $Tcirc^R$ that share common characteristics selected by the environmental pressures of susceptible

or resistant hosts, respectively. Fitness was calculated for Tcric^{S} and Tcirc^{R} following the F2 and F3 passages, and for the experiment in immune-suppressed lambs using the following calculation.

$$W = \frac{\text{Total number of L3 produced from days 21-50 dpi}}{\text{Infective dose of L3}}$$

The relative fitness (w) provides a comparative measure of the two isolates fitness. Using Tcirc^{S} as the control isolate, the fitness of the Tcirc^{S} genotype was normalized (w = 1) and the Tcirc^{R} fitness measured in respect to that, where:

$$w = W(Tcirc^{r}) / W(Tcric^{s})$$

Ratios ≤ 1 indicate greater relative fitness in Tcirc^{s}, ratios ≥ 1 indicate greater relative fitness Tcirc^{r}.

2.10 Statistical analysis

Statistical analyses using the SPSS software package were carried out using analyses of variance (one-way analysis of variance, ANOVA, and Mann-Whitney non-parametric test when data were over-dispersed). The life traits and fitness of Tcirc^{S} were compared against Tcirc^{R} following the F2 and F3 passage (where data was available) and following the experiment in immune-suppressed lambs. The life traits and fitness were also compared *within* each isolate, to explore if their performance differed significantly between the F2 and F3 passage. The FEC were log transformed (log x+1) prior to one-way ANOVA comparisons to stabilize the variance of the highly dispersed data.

3. Results

3.1 First Generation (F1) of Tcirc^{S} and Tcirc^{R}

The F1 generation of Tcirc^{S} and Tcirc^{R} originated from lambs with divergent FEC and worm burdens (Table 1). On average, the worm burden was 5-fold lower, and the FEC was 2-fold lower in resistant lambs compared to susceptible lambs.

3.2 Second Generation (F2) of Tcirc^{S} and Tcirc^{R}

Following the F2 passage, the relative fitness of Tcirc^{S} was significantly greater than Tcirc^{R} (Table 2). Due to the experimental limitations previously mentioned, comparisons of the isolate establishment and fertility were not possible. The FEC however, may serve as a proxy for establishment [25], and although not deemed significant, was 9 – fold lower in Tcirc^{R} than Tcirc^{S}.

3.3 Third Generation (F3) of Tcirc^{S} and Tcirc^{R}

Following the F3 passage, the establishment of Tcirc^{R} was significantly reduced compared to Tcirc^{S}. The relative fitness of Tcirc^{R} was also significantly lower than Tcirc^{S} (Table 2). Between the F2 and F3 passage, Tcirc^{R} significantly increased their egg to L3 larvae development ratio. Similarly, between the F2 and F3 passage, Tcirc^{S} significantly increased their fertility (Table 2).

3.4 Experimental Infection Comparing Tcirc^{S} vs. Tcirc^{R} in Immune-Suppressed Lambs

Following the experimental infection in immune-suppressed lambs, the establishment of Tcirc^{R} was significantly greater than Tcirc^{S}. The relative fitness of Tcirc^{R} was also significantly greater than Tcirc^{S} (Table 3).

4. Discussion

This study aimed to evaluate how exposure to a resistant host environment would alter the expression of life traits and infection dynamics of *T. circumcincta* when consequently infected in susceptible hosts. The results show that in as few as three generations of passage, the Tcirc^{R} isolate had become significantly more infective to susceptible lambs, with a greater establishment capacity and relative fitness compared to the control Tcirc^{S} isolate. This is the first evidence to indicate the resistant host environment can drive such rapid changes in the expression of GIN life traits. The potential biological processes facilitating these changes, and the implications on disease control, shall be discussed in turn.

Given the rapidity of the observed changes, it would seem unlikely that the increased establishment and fitness of the Tcric^{R} isolate would be attributable to genetic adaptation to the resistant environment in just three generations. Instead, the selective pressure exerted on these traits by the resistant ewes may have naturally selected phenotypes with a greater establishment capacity and consequent fitness. It is also possible that epigenetic changes were taking place. Phenotypic plasticity

281 in the expression of life traits is considered an essential survival mechanism of low mobility or sessile
282 organisms, such as nematodes, that cannot escape their habitat when it becomes unfavourable,
283 instead altering their phenotype to adapt to their changing habitat [24, 25, 26]. This has previously
284 been documented to take place within a single generation of the GIN *H. contortus*, where L3 larvae
285 exposed to desiccation conditions prior to experimental infection incurred a reduction in their
286 establishment capacity, they consequently compensated for this by increasing their reproductive
287 output, and ultimately maintaining comparable fitness levels to the control population [27]. Studies
288 with the free-living nematode, *Caenorhabditis elegans,* have also documented epigenetic changes to
289 take place across generations, where adults exposed to harsh environmental conditions produced
290 offspring that were better able to survive those conditions, matching the offspring phenotype to the
291 expected environment [25]. In the present study, we are not able to ascertain the exact biological
292 processes which lead to the observed increase in Tcric^{R} establishment and fitness when infected in
293 susceptible lambs, however, it seems that prior experience of the resistant host environment served
294 as the driver.

295

296 Several previous studies have explored whether reciprocal evolution could erode the benefits of
297 selective breeding with GIN overcoming the host protective responses. None of the studies found any
298 evidence in support of this for up to 30 generations of passage [28, 29, 20, 31, 32] with mathematical
299 models suggesting it would be a sustainable control option over the medium to long term [33]. Yet all
300 but one of these studies [31] relied on FEC as the sole indicator for GIN evolution, which potentially
301 overlooks changes that may have occurred elsewhere in the life traits. In the present study, the FEC
302 did not reflect alterations in Tcirc^{R} establishment or fitness. It would be of interest to repeat a similar
303 experiment, while including the additional measurements of life traits used in the present study, to
304 see if the GIN increased their fitness in resistant sheep with each successive passage. Between the F2
305 and F3 passage in resistant ewes, Tcric^{R} significantly increased their egg to L3 larvae development
306 ratio, and although not significant, nearly trebled their FEC and relative fitness value. This indicates an
307 increased performance of Tcric^{R} in the resistant ewes by the F3 generation. A series of experiments
308 which selected the GIN *Heligmosomoides polygrus* in resistant mice documented the isolate was
309 better able to survive and reproduce in immune hosts over time [34, 35].

310 The protective responses of resistant sheep can limit GIN success in different ways. Scottish Blackface
311 sheep for example, have been documented to indirectly negate *T. circumcincta* fertility by reducing
312 the size of the female worms [36]. The resistant ewes used in the present study did not impact the
313 Tcirc^{R} fertility, yet it would be pertinent to determine whether the different life traits targeted by

distinct immune mechanisms in the sheep were equally capable of a counter response, similar to that observed for establishment in the present study.

These results are potentially relevant to a farm scenario, specifically where selective breeding programs are harnessing similar genetic traits and GIN protective mechanisms across the flock. It is conceivable that the GIN population could encounter several resistant hosts in succession, modifying their infection dynamics for the more susceptible individuals on pasture, with greater associated health and production costs. There were however, experimental limitations to this study when extrapolating the results to field scenarios. Notably, the data were obtained from relatively few individuals that were immunosuppressed. Even within a sheep breed, individual variation in susceptibility to GIN infection has been well documented [37], thus, consequent studies using greater sample sizes would provide more accurate mean values to approximate what we could expect from the $Tcirc^{S}$ and $Tcirc^{R}$ infection dynamics in a field situation. By administering dexamethasone to the lambs prior to infection, we attempted to minimize individual variation in the lambs, where previous studies have demonstrated its efficacy in reducing natural resistance and immunity to GIN infections [16, 38]. This compound, however, has broad physiological uses [39] and it is not possible to guarantee a homogenous response across all lambs in the study. Further studies would be needed to verify whether resistant sheep, or indeed any GIN control tool that exerted a selective force on the population, would be capable of driving such rapid changes in the GIN infection dynamics on a farm-level. This work highlights that under experimental conditions, it is possible, and anticipating the evolutionary consequences of any control intervention is crucial to ensuring its long-term viability and responsible application.

Declaration of Competing Interests

None.

Author Contribution

Conceptualization: JC and LG. Funding: ES. Project administration: JC. Methodology, data collection: ES, JC, LG. Data analysis and interpretation: ES, CC, JC. Writing: CC, JC. Review and Editing: CC, ES, JC.

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Table 1. F1 generation of Tcirc^{S} and Tcirc^{R}: faecal egg count and establishment in susceptible and resistant lambs.

	FEC	Establishment
Tcirc^{s} producing lambs		
Susceptible lamb 1	210	4128
Susceptible lamb 2	410	4297
Tcirc^{r} producing lambs		
Resistant lamb 1	50	600
Resistant lamb 2	150	800

FEC: faecal egg count, calculated as eggs per gram of faeces (EPG)

494 Table 2. F2 and F3 generation of Tcirc^{S} and Tcirc^{R}: Life traits and fitness following passage in
495 susceptible and resistant sheep.

	Tcirc^{S} passaged in susceptible lambs	**Tcirc^{R} passaged in resistant ewes**	**Significance: Tcirc^{S} vs. Tcirc^{R}**
Establishment			
F2 passage	37 ± 8	NA	
F3 passage	45 ± 2	21 ± 9	*
Fertility			
F2 passage	247 ± 54^{a}	NA	
F3 passage	476 ± 29^{b}	123 ± 60	
Egg – L3 larvae development ratio			
F2 passage	0.72 ± 0.40	0.42 ± 0.81^{a}	
F3 passage	0.99 ± 0.19	0.71 ± 0.62^{b}	
FEC			
F2 passage	448 ± 364	51 ± 60	
F3 passage	396 ± 180	146 ± 118	
Relative fitness ratio (w)			
F2 passage		0.21 ± 0.75	*
F3 passage		0.70 ± 0.30	*

496 Life traits are expressed as mean values ± standard deviation. Establishment: percent adults from
497 infective of dose. Fecundity: = EPGavg x QFM / Number of females. Egg – L3 larvae development ratio:
498 L3 per gram faeces / EPG. FEC: number of eggs per gram (EPG) of faeces. (NA) No available data
499 following the F2 passage in resistant ewes for establishment and fertility. Relative fitness (w) = W Tcirc^{R}
500 / W Tcirc^{S}; where ratios ≤ 1 indicate greater fitness in Tcirc^{S}, ratios ≥ 1 indicate greater fitness Tcirc^{R}.
501 Significant differences *between* the isolates Tcirc^{s} and Tcirc^{r} are denoted with *, where ANOVA $P \leq 0.05$.
502 Significant differences *within* the isolates, between the F2 and F3 passages, denoted with different
503 letters in superscript, where ANOVA $P \leq 0.05$.

Table 3. Comparison of life traits and fitness in Tcirc^{S} vs. Tcirc^{R} following experimental infection in immune-suppressed lambs.

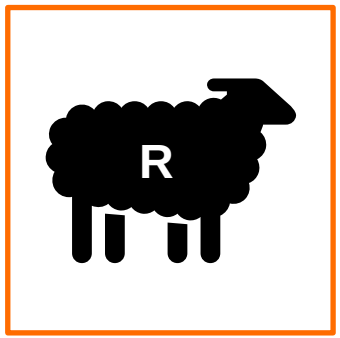
	Tcirc^{S}	**Tcirc^{R}**	**Significance Tcirc^{r} vs. Tcirc^{s}**
Establishment	35 ± 6	48 ± 4	*
Fertility	214 ± 41	187± 77	
Egg – L3 larvae development ratio	1.02 ± 0.19	1.39 ± 0.92	
FEC	200 ± 70	181 ± 72	
Relative fitness ratio (w)		1.39 ± 0.41	*

Life traits are expressed as mean values ± standard deviation. Establishment: percent adults from infective of dose. Fertility: = EPGavg x QFM / Number of females. Egg – L3 larvae development ratio: L3 per gram faeces / EPG. FEC: number of eggs per gram (EPG) of faeces. Relative fitness (w) = W Tcirc^{R} / W Tcirc^{S}; where ratios ≤ 1 indicate greater fitness in Tcirc^{S}, ratios ≥ 1 indicate greater fitness Tcirc^{R}. Significant differences between the isolates Tcirc^{S} and Tcirc^{R} are denoted with *, where ANOVA $P \leq 0.05$.

Gastrointestinal Nematodes (GIN): Changing Infection Dynamics

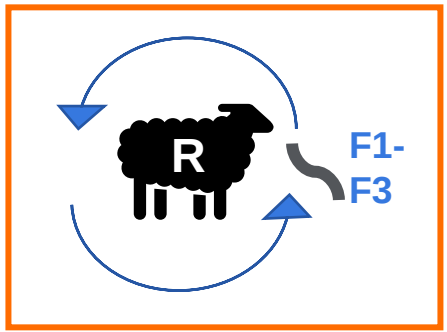
Passage through resistant hosts drives parasite infectivity in susceptible hosts

Sheep selectively breed for GIN resistance



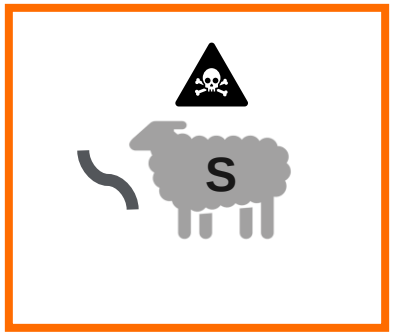
Parasitology International

Selected a GIN isolate over 3 generations



Chylinski C, Schmidt E, Gruner L, Cabaret J

That was more infective in susceptible sheep



Serial passage in resistant sheep