

Arrested development of *Trichostrongylus colubriformis* in experimentally infected rabbits. Effect of decreasing photoperiod, low temperature and desiccation

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Summary

Inhibited development of *Trichostrongylus colubriformis* was studied in rabbits. Experiments are described which show that in a recently isolated field strain of the nematode, the primary stimulus to the induction of inhibited development at the early third larval stage (EL₃) in the host is exposure of infective larvae and hosts at decreasing photoperiod. Thus exposure of infective larvae and hosts to photoperiod decreasing daily by 5 min. for 4 weeks prior to their oral administration to rabbits resulted in a mean of 95.2 % of the established worm burden becoming inhibited at the EL₃ stage. If only the L₃ stages were exposed to decreasing photoperiod prior to infection of rabbits, then the prevalence of inhibited EL₃ stages amongst the worms established declined. Infective larvae subjected to periods of 4 weeks at 4°C and at the same time at decreasing photoperiod were able to induce arrested development in 90.6 % of the larvae.

Storage of infective larvae at 4°C as well as storage at desiccation state for 4 weeks before being given as single infections to rabbits did not increase the proportion of worms subsequently found to be hypobiotic.

Key words: *Trichostrongylus colubriformis*; hypobiosis; arrested development; photoperiod; low temperature; desiccation

Introduction

Hypobiosis, arrested and inhibited development are synonymous terms used to describe the cessation of development at an early phase of parasitic existence in the host. It is a common phenomenon in parasitic nematodes, particularly in species of the family Trichostrongylidae, Strongylidae and Chabertiidae and plays an important role in the epidemiology of nematode infections. Extensive reviews have been written by Michel (1974), Schad (1977), Gibbs

(1986), Armour and Duncan (1987). Eysker (1993, 1997) produced a relatively recent review on this phenomenon in trichostrongylids in ruminants. The phenomenon occurs in a wide variety of host-parasite relationships and for *Trichostrongylus* spp. it is the early 3rd stage (EL₃), which becomes inhibited or arrested. Inhibited larvae have a low metabolism and can be recognised by their uniform size. This enables them to survive for long periods, after which they may resume development.

Inhibited development is a complex phenomenon which is not completely understood and in gastrointestinal nematodes of ruminants seasonal climatic conditions and host resistance are considered to be the most important factors involved. The finding of a high degree of hypobiosis in non-immune tracer animals in many studies suggests that environmental factors rather than immunological responses may regulate the phenomenon.

The seasonal onset of hypobiosis of trichostrongylids of ruminants in the temperate areas of the northern hemisphere is associated with decreasing temperatures and photoperiod, suggesting that these factors may be important stimuli acting on the free-living stages of the nematode (Fernandez *et al.*, 1999).

However, earlier studies specifically conducted on *Haemonchus* spp., *Ostertagia* spp., *Cooperia* spp. or on *Obeliscoides cuniculi*, a trichostrongylid of snowshoe hares, demonstrated discrepancies in maintenance of the third stage larvae at low temperatures (Fernando *et al.*, 1971; Armour and Bruce, 1974; Smeal and Donald, 1982; Gibbs *et al.*, 1977; Bisset, 1983; Mansfield *et al.*, 1977; Zinsli, 1987) and in various, mostly constant, photoperiod regimes (Connan, 1975; Smith, 1977; Gibbs, 1982; Chiejina *et al.*, 1988; Capitini, 1990).

The following experiments were carried out to investigate the causes of hypobiosis in *Trichostrongylus colubriformis*. Because the use of substantial numbers of large farm ani-

mals in experimental studies can be very expensive we studied it in rabbits as model animal. The domestic rabbit has been shown to be a good host for *T. colubriformis* (Bezúbik *et al.*, 1988, Wedrychowicz and Bezúbik, 1988).

The study focussed on the effects of artificially induced low temperature, decreasing photoperiod and desiccation on infective larvae of this species in an attempt to simulate autumnal conditions of temperature and photoperiod in Czech Republic.

Materials and Methods

Two 1/2 Bursian x 1/2 Czech short hair white crossbred male goat kids were treated per os with 10 mg kg⁻¹ fenbendazole (Panacur, American Hoechst Corp.) at 2 months of age. Faecal examinations at the time of treatment and 1 week later were negative for helminth eggs. The goats were each infected with 5 000 freshly cultured L₃ of a field strain of *Trichostrongylus colubriformis* on July 1999. Infective larvae required for conditioning were obtained by collecting faeces directly from goats over a week period, from July 24 to July 31. The faeces were cultured at 23°C for 10 days. Infective larvae were recovered by Baermann extraction.

Batches of L₃ in water (LB) containing 5 – 8 000 L₃/dish were subjected during 4 weeks to different laboratory conditions, namely:

LB 1, 23°C, in photoperiod decreasing daily by 5 min., from 10 hrs to 7 hrs and 40 min. Light intensity was attained by means of thermostat by extension cord fitted with 8 watt fluorescent tube automatically switched on and off by timers.

LB 2, 4°C, in similarly decreasing photoperiod. Light intensity was provided within the refrigerator by extension cord fitted with 8 watt fluorescent tube automatically switched on and off by timers.

LB 3, 4°C, in constant photoperiod (12 hrs of darkness)

LB 4, 20°C, in a state of desiccation, in constant photoperiod (12 hrs of darkness).

Each larval batch was disposed in 2–4 Petri dishes. The water level was checked daily.

50 rabbits were allotted to 10 groups of 5 rabbits of similar age, weight and gender. Following a 2-day acclimation period, the rabbits were switched from the commercial rabbit chow to a modification of the commercial chow containing prednisolone (Prednison, 80 mg kg⁻¹ day⁻¹) for the duration of the study.

The course of *T. colubriformis* infection in rabbits resembles that in ruminants (Bezúbik *et al.*, 1988) and rabbits produce both systemic and local humoral responses to *T. colubriformis* infection (Wedrychowicz and Bezúbik, 1988). The immunosuppression on the rabbits with continuous administration of prednisolone showed no significant increase in the propensity of *Trichostrongylus* larvae for arrested development (Schmid, 1986).

Until they were infected, parasite-naive rabbits were kept in the laboratory boxes under two photoperiod regimes, either under constant photoperiod regimes (12 hrs of dark-

ness) or the rabbits were exposed to the autumn photoperiod regimes for one month (photoperiod decreasing daily by 5 min., from 10 hrs). The rabbits were infected with standard dose of 750 L₃ (except Group 8) to minimize the reaction of host immunity. Design of experiments is presented in Table 1.

The rabbits were necropsied 4 weeks after infection. Gastric and intestinal ingesta were decanted and larvae and adult parasites were counted. The larvae inhibited in development were released from the wall of stomach and intestine by means of digestion of mucosa in the solution of pepsin and hydrochloric acid (13 g pepsin, 10.8 ml HCl in 1000 ml of distilled water) for 4 hrs at 39°C. Parasites were recovered by vigorously rubbing and washing the intestinal mucosae several times in water. The solution was decanted overnight and larvae were counted.

Due to binomic distribution the effect of factors was analysed by weighted logistic regression within generalised linear models (GLM). In each case, the mean percentage composition of arrested larvae recorded in the various treatment groups were compared with those recovered from control rabbits and examined for statistically significant differences by the chi-square test. In addition, the least significant difference (LSD) at the 1, 5 and 10 % level was calculated (Crawley 1993).

Results

The number of adult worms and early L₃ (EL₃) recovered from each of groups of the rabbits inoculated with *Trichostrongylus colubriformis* L₃ subjected to different temperatures, photoperiods and state of desiccation are summarized in Tables 1 and 2.

Decreasing photoperiod (Groups 1, 2, 3)

In order to determine whether there are differences in the influence of the factor on the induction of hypobiosis, the influence of the factor on host and parasite are tested separately.

Table 1 indicates that the decreasing photoperiod at which the larvae are stored for 4 weeks influences the subsequent development in the rabbit. The mean percentage of EL₃ found in group 1 and 3 were 53.3 % and 95.2 %, respectively, compared to nil (Group 4) or very low (Groups 7,8) EL₃ in the case of L₃ either stored at low temperature or in desiccated state (Group 9). Inoculation of freshly harvested L₃ did not give rise to inhibited EL₃ populations in the rabbits exposed to the autumn photoperiod regimes for one month prior to inoculation (Group 2). The difference between EL₃ counts in the control and groups 1 and 3 was statistically significant.

Decreasing photoperiod and low temperature (Groups 4, 5, 6)

The pattern of the increase in the proportion of retarded larvae obtained when larvae were stored at 4°C and in photoperiod decreasing daily by 5 min. (Groups 5,6) was approximately similar to that obtained with larvae stored at

Table 1. Number of *Trichostrongylus colubriformis* recovered from rabbits infected 20 days previously with 750 (except group 8 receiving 3750) live infective larvae (L₃) either conditioned or freshly harvested

| Group | Exposure to light ¹ | | | Total | Worm counts (±SEM) | | |
|--------|--------------------------------|-------------------------------------|---|----------|--------------------|-----------------|--------------------------------|
| | Host | L ₃ Light | L ₃ Temperature ³ | | Adult | EL ₃ | EL ₃ % ⁵ |
| 1 | Constant | Decreasing | 23°C | 8 (4) | 1 (1) | 4 (0) | 53.3 |
| 2 | Decreasing | Fresh ² | – | 44 (24) | 41 (22) | 0 | 0 |
| 3 | Decreasing | Decreasing | 23°C | 49 (42) | 1 (2) | 47 (43) | 95.2 |
| 4 | Decreasing | Constant | 4°C | 23 (9) | 21 (7) | 0 | 0 |
| 5 | Constant | Decreasing | 4°C | 83 (124) | 7 (4) | 75 (121) | 90.6 |
| 6 | Decreasing | Decreasing | 4°C | 21 (2) | 10 (3) | 9 (3) | 40.9 |
| 7 | Constant | Constant | 4°C | 18 (9) | 16 (10) | 1 (3) | 6.6 |
| 8 | Constant | Constant | 4°C | 21 (11) | 15 (7) | 3 (2) | 13.4 |
| 9 | Constant | Constant (desiccation) ⁴ | 20°C | 18 (12) | 15 (13) | 2 (3) | 9.5 |
| 10 (C) | Constant | Fresh ² | – | 51 (47) | 47 (46) | 2 (3) | 3.2 |

¹ Type of light/dark regime exposed to for 4 weeks; ² Rabbits infected with freshly collected L₃; ³ Temperature at which the L₃ were kept; ⁴ L₃ kept in a state of desiccation for 4 weeks; ⁵ %'s calculated from mean values, before being rounded off; EL₃ = early L₃

Table 2. Comparison of relative frequencies (F) of EL₃ in the experimental groups. The frequencies were mutually compared using the least significant difference (LSD) test: P < 0.1 (#), P < 0.05 (\$) a P < 0.01 (+). N = number of EL₃ recovered from all rabbits in a group. * = a group with "0" frequency. In order to perform an analysis the value was replaced by "1". NS = no significant differences

| Group | F | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | C |
|-------|-------|---|----|----|----|----|----|----|----|----|----|
| 1 | 0.53 | | NS | # | NS | NS | NS | NS | NS | NS | # |
| 2* | 0.004 | | | \$ | NS | \$ | NS | NS | NS | NS | NS |
| 3 | 0.95 | | | | NS | \$ | \$ | NS | NS | NS | + |
| 4* | 0.02 | | | | | NS | \$ | NS | NS | NS | NS |
| 5 | 0.91 | | | | | | \$ | + | NS | NS | + |
| 6 | 0.41 | | | | | | | NS | NS | NS | \$ |
| 7 | 0.07 | | | | | | | | NS | NS | NS |
| 8 | 0.13 | | | | | | | | | NS | NS |
| 9 | 0.10 | | | | | | | | | | NS |
| K | 0.03 | | | | | | | | | | |

23°C and under autumn photoperiod regimes (Groups 1 and 3). There was a progressive increase in the percentage inhibition at EL₃, the maximum percentage inhibition was 90.6 % (Group 5). The proportion of retarded larvae was nil when larvae were stored at 4°C and in constant photoperiod and hosts were exposed to the autumn photoperiod regimes (Group 4). Statistically significant differences occurred between proportions of the EL₃ of control group and group 5 and group 6 (Table 2).

Low temperature (Groups 7,8)

Results shown in Table 1 indicate that the exposure of infective *T. colubriformis* larvae at low temperature (4°C) for 4 weeks has no significant effect on the intrinsic pattern of development of the population.

Larvae stored for 4 weeks under this condition (Group 7) showed 6.6 % (infection dose 750 L₃) and 13.4 % (infection dose 3750 L₃) retardation (Group 8).

Desiccation (Group 9)

Exposure of third stage larvae of *T. colubriformis* to desic-

cation for 4 weeks produced an infection that contained 9.5 % retarded larvae.

Control (Group 10)

Control infections with non-conditioned larvae resulted in a low number of inhibited larvae in all rabbits (3.2 %).

Discussion

The main objective of this investigation was to define those factors that may act on the free-living third stage larvae (L₃), causing them to undergo a hypobiotic phase as early third-stage larvae (EL₃) in the gastric or intestinal mucosa of the rabbits.

The exact etiology of inhibition in larval nematodes is obscure but there has been considerable speculation on its mechanism. Initially, hypobiosis was thought to be associated with acquisition of immunity (Martin *et al.*, 1957; Michel, 1963; Soulsby, 1966). Later this theory was, however, questioned since hypobiosis was found to be independent of the length of time grazed and previous infec-

tions (Anderson *et al.*, 1965; Armour *et al.*, 1969b). The non-immune inhibition in nematode larvae has been reviewed by Armour and Bruce (1974), Michel (1974), Schad (1977), Gibbs (1982), Anderson (1988), Eysker (1993, 1997) and, as cited, this phenomenon could be ascribed to inherent developmental changes in the infective larval stages, either genetically or environmentally induced (Armour *et al.*, 1969 a, b; Eysker, 1997).

The findings of the present paper support the supposition that the exposure of infective larvae and the hosts to environmental factors such as decreasing photoperiod prior to infection influences the proportion of worms that become inhibited at early third-stage. This fact would indicate the importance of season (autumn) as an inductive factor of inhibition or hypobiosis over any other factor. The combination of factors as decreasing photoperiod and low temperature simulating autumn conditions (represented by Group 5) seems to be the trigger that acts on most of the infective L₃, inducing them to an inhibited stage. When considering both factors independently, it can be noted that decreasing light had a stronger influence than the low temperature exposure time (Group 3).

Though the photoperiod has been considered one of the possible environmental factors that leads to the accumulation of hypobiotic larvae (Michel *et al.*, 1973a,b; Smith, 1974, 1977; Nari *et al.*, 1982; Capitini, 1990), this has been tested in very few trials. Gibbs (1973) found that larvae of *H. contortus* exposed to a short photoperiod (10 hrs) as opposed to a long photoperiod (16 hrs), or fresh larvae, showed a significant level of arrest (70 %). However, Smith (1977) and Chiejina *et al.* (1988) were unable to confirm the effects of the length of day on trichostrongylids. Connan (1975) observed an increase in the hypobiosis of the *H. contortus* which was inhibited established from larvae cultured in the permanent dark. However, the intensity of hypobiosis of *Trichostrongylus* spp. did not exceed 26 % of the worm populations (Ogunsu and Eysker, 1979).

It has also been suggested that host effects might be responsible for affecting the arrested development of the trichostrongylids. On the premise that since photoperiod can influence host physiology behaviour (Ortavan *et al.*, 1964) which might in turn influence nematode parasite behaviour, Blitz (1970) conducted an experiment using two groups of ewes infected with *H. contortus*. These two groups of ewes were subjected to two photoperiod regimes, one using a fixed long day of 18 h of light and 6 h of darkness and the other the prevailing environmental photoperiod. Larvae from these ewes which were dosed to groups of parasite-free lambs held under similar conditions of photoperiod showed no significant differences in rate of arrest between groups.

However, while a great deal of work has been done with constant photoperiod, hypobiosis seems to be related to the onset of changeable photoperiods. Nari *et al.* (1982) found that *O. circumcincta* larvae arrested under progressive decreasing photoperiod showed higher percentages (53.1 %) of hypobiosis than those exposed to increasing photoperiod

(0.31 %) or those exposed to fixed photoperiod (2.33 %). In Scotland, Armour (1978) studied *O. ostertagi* in cattle by artificially decreasing the day length (although it was always combined with temperature 2 – 9°C) and concluded that the photoperiod seems to have an influence on third-stage larvae. The studies cited above were carried out in areas where arrested larval development occurs in the autumn and winter seasons. In relation to the contrasting conditions of the Southern hemisphere, Fernandez *et al.* (1999) presented trial that indicates that the increasing temperature and increasing time of light exposure simulating spring conditions, acting on *O. ostertagi*-infective larvae is one of the initial triggers for the larval inhibition for the Argentinean cattle production system.

Temperature has received greater attention in previous literature. The studies carried out in Scotland (Armour and Bruce, 1974; Armour, 1978) and in England (Michel *et al.*, 1974, 1975) with *O. ostertagi* or Fernando *et al.* (1971), Gibbs *et al.* (1977) with *O. cuniculi*, showed that the arrest during early parasitic development was induced by maintenance of the third stage larvae at low temperatures. On the other hand, Bisset *et al.* (1983) and Mansfield *et al.* (1977) showed that exposure of trichostrongylid third stage larvae to these conditions induced no significant arrested development.

Smeal and Donald (1981) concluded that inhibition-proneness was inherent in the population and drew attention to the evidence from British findings that a high proportion of *O. ostertagi* infective larvae of such populations become inhibited in development by responding to environmental stimuli (Smeal *et al.*, 1980). In contrast, the Australian populations of *O. ostertagi* showed no effective response (very low rate of inhibition) after 8 weeks of larval storage at 4°C (Smeal and Donald, 1982). The weak response to a cold stimulus reported may represent the partial retention of ancestral European behaviour by the parasite, despite its adaptation to different stimuli in Australia, as was suggested by Michel (1978).

Hypobiosis has been studied less in tropical climates than in temperate regions, where falling temperatures and photoperiods have confirmed to trigger this phenomenon. In tropical climates, although the exact cause of hypobiosis is not known, it is believed to be related to dry conditions (Pandey, 1990; Eysker and Pandey, 1989; Ndao *et al.*, 1995). The present data indicate the low occurrence (9.5 %) of arrested development of Czech *T. colubriformis* (Group 9). It seems therefore that the degree of this environmental adversity (desiccation) may not be important in this certain strain of nematode for this hypobiosis. The present work indicates that the strain of *T. colubriformis* prevalent in Czech goats is not sensitive to desiccation, as regards the development of hypobiosis.

Only very little is reported about hypobiosis of *Trichostrongylus* spp. Ogunsu and Eysker (1979) did not observe any significant hypobiosis with *Trichostrongylus* spp. from sheep in northern Nigeria. However, Eysker (1978) found considerable inhibition of *Trichostrongylus* spp. in ewes, in contrast, to very limited arrested develop-

ment in lambs. According to Michel (1952 a, b), large proportion of the *T. retortaeformis* larvae given to previously infected rabbits may fail to develop beyond the late third stage. Burdens of adult *T. retortaeformis* are regulated by the occurrence of an abrupt elimination of fifth stage worms whenever the biomass of worms exceeds a critical level.

Vladimirova (1982), Altaif and Issa (1983), Ndao *et al.* (1995) reported about EL₄ of *T. colubriformis*. Exposure of infective larvae for 7 weeks to 4°C in winter time induced the high level of retardation (L₄) but this conditioning in summer was much less effective (Vladimirova, 1982).

The use of *T. colubriformis* and rabbit system as a possible simulant of trichostrongylidosis in ruminants is suggested by a number of similarities in host-parasite relations. Bezubik *et al.* (1988) reported that course of *T. colubriformis* infection in rabbits resembled that in the ruminants. Also, the immunization of the rabbits with two doses of 20 000 infective larvae induced immunity against subsequent infection to a degree similar to that observed in sheep.

Based on the relatively high proportions of early third stage parasitic larvae recovered from the rabbits in the present experiment, it is likely that the decreasing photoperiod, rather than temperature was responsible for the hypobiosis observed. Nevertheless, the importance of host has been studied and will be the subject of a further report.

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