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The development and overwintering survival of free-living larvae of *Haemonchus contortus* in Sweden

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Abstract

Five complimentary studies were undertaken with the overall aim to examine the ability of free-living stages of *Haemonchus contortus* to over-winter and tolerate cold stress. Two studies deal with the development and long-term survival of eggs and infective larvae of two geographically different isolates (Kenya and Sweden). Eggs and larvae were monitored in climatic chambers at temperatures that fluctuated daily between −1°C and 15°C, or at constant temperatures of 5°C and 15°C. The development from egg to larvae was dependent on temperatures over 5°C. The long time survival was favoured at lower temperatures. Furthermore, the overwintering capacity of the free-living stages of these isolates was estimated under Swedish field conditions. Two groups of lambs were experimentally infected with different isolates, and kept separated on previously ungrazed plots. In early May the following year, two parasite-naive tracer lambs were turned out on each of the plots to estimate the pick up of overwintered larvae. This experiment was replicated in central and southern Sweden. In addition, two experiments were performed in 2003 on pasture previously grazed by naturally infected sheep. One trial was on a pasture in southern Sweden grazed by a commercial flock, where extreme numbers of *H. contortus* were found towards the end of the grazing season 2002. The other study was on a pasture plot in central Sweden grazed by a hobby flock in 2002, where three of six lambs died due to haemonchiasis. Overwintered *H. contortus* was recorded on three of four experimental sites. Worm burdens were in all instances extremely low. No differences in development and survival were found between the isolates. Consequently, overwintering on pasture is of no practical significance in the transmission of *H. contortus* between grazing-seasons in Sweden.

Introduction

Haemonchus contortus is a nematode parasite of small ruminants, which is of major economic and animal welfare importance to the sheep industry worldwide. The life cycle of *H. contortus* involves a free-living period when the egg and larval stages are exposed to the environment outside of the host and factors such as temperature and moisture are of great importance for the hatching, development and survival of the parasitic eggs and larvae (Levine, 1963). Thus, outside environmental conditions have a major impact on the parasite population. In general, *H. contortus* is regarded as being well adapted to a warm and humid climate. Many studies have investigated the environmental variables that determine the success of the development process of *H. contortus* eggs to infective larvae. However, the situation is essentially unchanged since the pioneering work of Gordon (1948), who described the lower environmental limits for haemonchiasis in sheep as a

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mean monthly temperature of 18°C and a minimum monthly rainfall of two inches. Although little or no development of eggs will take place below 9°C (Silverman & Campbell, 1959), this nematode also occurs in cool temperate regions.

In Sweden, haemonchiasis is an emerging problem in sheep and goat production, although the external environmental conditions for the development of free-living stages on pasture are only favourable for short periods of time during the grazing season (Lindqvist *et al.*, 2001). In a recent Swedish survey, the northernmost finding of *H. contortus* was discovered near the Arctic Circle (Lindqvist *et al.*, 2001). Sweden's climate is predominately influenced by its northern latitude, between 55° and 69°N, and the Gulf Stream. The climate varies considerably throughout the country because of its expansive north–south gradient, but is considered temperate, due to the mild and wet Atlantic winds that prevail along the country's western border with Norway. Winters may be bitterly cold and Sweden experiences heavy frosts and plentiful snowfalls during the winter months (Smith, 2000). Accordingly, it could be speculated that *H. contortus* in Sweden has become adapted to cold conditions.

In earlier studies on the overwintering survival of nematode parasites of ruminants in the Nordic countries, it was proposed that there is a positive relationship between the severity of the winter and the survival of free-living stages of the parasites on pasture, due to the buffering capacity of a snow cover (e.g. Tharaldsen, 1976). Nevertheless, based on the assumption that free-living stages of *H. contortus* are unlikely to survive both the severe weather conditions and the length of time that sheep are housed (generally from mid October to late April) in Sweden, the current advice given by the Swedish Animal Health Service to sheep farmers is that pastures will be free of *H. contortus* after one winter if sheep are housed, provided they are treated with a highly effective anthelmintic during the housing period (Rudby-Martin, 2002).

The epidemiological importance and possible climatic adaptations of *H. contortus* have stimulated our interest in the investigation of overwintering survival of this parasite on pasture in Sweden. In the present paper, results are described from several complementary studies. Two of these were performed under controlled conditions in climatic chambers, where the development and survival of eggs and larvae from isolates of *H. contortus* from Kenya and Sweden were investigated. These isolates have been shown to be genetically distinct, based on differences in the internal transcribed spacer 1 (ITS-1) of the rRNA, but the differences were not found to be fixed and the isolates were therefore regarded as belonging to the same species, *H. contortus* (Troell *et al.*, 2003). Nevertheless, these represent two genetically separated populations of *H. contortus* originating from two environmental extremes and it can therefore be assumed that their free-living stages have been subjected to different selection pressures in the external environment.

Three studies were field-based in central and southern Sweden, using the tracer lamb technique to determine whether the pick-up of *H. contortus* occurred on spring pastures that were grazed the previous year by sheep infected with *H. contortus*.

Materials and methods

Parasite isolates

The Kenyan isolate originated from a farm on the Kapiti plains, eastern Kenya. Since 1998, it has been maintained in experimentally infected sheep at the International Livestock Research Institute (ILRI) in Nairobi. Infective larvae from this source were consigned to Sweden in September 2000 and two helminthologically naïve young lambs were infected. Larvae recovered from faecal cultures of these animals were stored at 10°C for 32 weeks, before further passage in worm-free lambs. Field studies were based on larvae from the first passage in Sweden, whereas laboratory studies were conducted with faeces from lambs infected with larvae from the second passage that had been stored at 5°C.

The Swedish *H. contortus* isolate was initially isolated in 2000 by the recovery of approximately 200 adult *H. contortus* from naturally infected sheep which were surgically transferred directly into the abomasum of worm free lambs. This isolate originated from a farm in Västernorrland (latitude 63° N), in northern Sweden.

Comparison of Haemonchus *isolates*

Development from eggs to larvae

The development and survival of eggs from these two genetically distinct isolates of *H. contortus* were monitored at three temperatures, i.e. 5°C and 15°C or daily fluctuation between −1 to 15°C, in climatic chambers. Faeces were collected from four lambs maintained indoors with experimental monospecific infections for each *H. contortus* isolate. At 35 days post-infection, faeces from lambs infected with the same isolate were thoroughly mixed and aliquots of 10 g containing approximately 3000 epg were dispensed in plastic beakers. In order to simulate the thermal conditions at the time of turn-out in mid spring in Sweden, the beakers were kept in a climatic chamber (Firlabo CV100 FHPN20) at maximum humidity in a chamber where the temperature fluctuated daily between −1 to 15°C. Beakers with faeces were also kept at constant temperatures of 5°C and 15°C in two separate climatic chambers (Nüve, ES 110 cooled incubator). For each isolate, four quadruplet beakers (in total 16) with faeces were cultured at each temperature regimen. Faeces were incubated for 2, 3, 4 and 5 weeks and then examined with respect to the development and survival of eggs and larvae. At each time interval, larvae in two beakers from each isolate were harvested and enumerated directly, whereas two beakers were incubated for an extra week at 25°C, to enable all viable free-living stages to develop to the infective third larval stage. In addition, eight faecal cultures maintained at 25°C for 10 days were used as a positive control.

Long-time survival of infective larvae

Third stage larvae (L3) were cultured at room temperature for 10 days from faeces from experimentally infected animals. Larvae from each of the Swedish and Kenyan isolate were cultured and handled separately to avoid the risk of cross contamination. The L3 were harvested and stored in flat-sided cell culture flasks. One flask for each harvest was used, totalling six flasks for each temperature. Each flask contained approximately 70 000 larvae in 20 ml of 1:1 PBS, water and Fungizon (0.25 mg l^{-1} PBS, pH 7.5). Flasks were maintained at three temperatures (constant at 5°C, 15°C, or daily fluctuation between −1 and 15°C) in environmental chambers as described above. Larval survival was examined after 4, 6, 8, 12, 16 and 24 weeks. Larvae were kept on the bench for 1 h to allow them to adjust to room temperature, and then counted in four aliquots of 25 μl under a stereomicroscope. The total numbers of motile larvae were estimated.

Tracer studies

Three tracer-lamb studies were performed during the years 2002 and 2003 (table 1). Temperature data were obtained from the Swedish Meteorological and Hydrological Institute (SMHI) and were collected at meteorological stations 7–48 km from the experimental sites.

Malmö and Lövsta

Experiments were set up in the summer of 2001. One study was performed outside Malmö, Skåne, located in south-western Sweden. The other site was in Lövsta, Uppland, in mid-eastern Sweden. At each location, the experiment was replicated by the use of two plots of 0.8 ha each. In Malmö, sheep had not grazed the plots within the last seven years. In Lövsta, first-season grazing sheep had been allocated to pastures for the first 6 weeks (April–May) of the grazing season in 2000, but for the remaining season were unstocked. Additionally the farm at Lövsta had never reported *H. contortus* in their sheep.

On each site, two lambs and two companion ewes were placed on each plot, giving a total of four plots and 16 animals. A minimum of 4 weeks prior to larval infection, all ewes at both sites were treated with a subcutaneous formulation of ivermectin (Ivomec®, MSD) at the standard dose rate of 0.2 mg kg^{-1} body weight. Lambs were 6 months old and were untreated as they had previously never been out on pasture. The animals were turned out on July 7 after all ewes and lambs had been experimentally inoculated with a single dose of 1500 infective larvae of either Kenyan or Swedish origin. The larval dose was dispensed orally to each individual in a small volume of water. The larvae used for infection were cultured from faeces and had been kept at 10°C up to 6 months prior to infection.

Faecal egg counts, conducted 6 weeks after the sheep had been allocated to the pastures, showed that they were all infected, thus the pastures were contaminated from mid summer to late October 2001. Faecal samples were examined by a modified McMaster technique (Gordon & Whitlock, 1939). Sheep grazed the experimental pastures for 5 months. All animals were slaughtered at the time of housing.

At the time of turnout the following year (6 May 2002), two lambs (4 months of age) and two companion ewes (treated with ivermectin, standard dose, 7 days prior turnout) were released on to each plot. To confirm that all animals were not infected with nematode parasites, faecal samples were taken at turnout. Faecal samples were examined again three weeks after turnout and thereafter weekly until slaughter. After 6 weeks on pasture, the lambs were slaughtered and at autopsy the content of the abomasum from each lamb was collected, the abomasal mucosa was washed carefully, inspected macroscopically and the total collection was passed through a series of sieves with the finest mesh of 250 μm. The retained material on all the sieves was preserved in 50% ethanol and refrigerated. This was examined in small portions under a stereomicroscope and all adult worms recovered were differentiated into species and sexed.

Bergshamra

This experiment was set up in the spring of 2003 at Bergshamra in central Sweden, on a 1.25 ha plot where approximately 30% of the ground was covered by grass. Throughout the summer of 2002, three ewes (previously untreated with anthelmintic) and six lambs grazed this pasture, which had never been used for sheep. At the end of the grazing season, the pasture was considered as being heavily contaminated, due to the finding of a massive *H. contortus* infection in a lamb that died and was subsequently sent for autopsy to the Swedish National Veterinary Institute in September 2002. The lamb was the third to die in the flock. The results of the autopsy confirmed severe clinical haemonchiasis. The lamb harboured at time of death 22, 000 epg, which were almost entirely *H. contortus*.

The area was previously covered by forest and had until 2000 never been grazed by domesticated animals. Accordingly, any parasites on these pastures must have originated from the three ewes that were turned out in early June 2002.

Table 1. Information on the experimental sites used during 2001–2003. Sites 1 and 2 were used for experimental infections and 3 and 4 were conventional sheep farms used for field studies.

Site	Year	Location	Latitude	Background
1	2002	Southern Sweden (Malmö)	55°N	Experimentally infected during 2001
2	2002	Central Sweden (Lövsta)	59°N	Experimentally infected during 2001
3	2003	Central Sweden (Berhshamra)	59°N	Naturally infected, 2002 only
4	2003	Southern Sweden (Vomb)	55°N	Naturally infected, 2002

At the time of turnout the following year (15 May 2003), the same pasture was stocked with six parasite-free tracer lambs and two companion ewes. All lambs were born in February 2003 and had not been on pasture before turnout, and the ewes were treated with doramectin (Dectomax vet.®, Pfizer) at the standard dose rate of 0.2 mg kg^{-1} bodyweight during the previous housing period. The tracer test period was 20 days, and lambs were then housed for an additional three weeks to prevent auto-infection and to allow for recently acquired larvae the opportunity to develop into adult worms.

Faecal samples were taken from all animals at turnout, and from the tracer lambs one week after housing as well as at slaughter. At slaughter, the abomasum, abomasal mucosa, and small intestine were examined separately, according to the procedures described by Donald *et al.* (1978). Inhibited larvae (early L4) were recovered from acid-pepsin digestion of the abomasal mucosa. The digestion proceeded according to standard practices (Dobson *et al.*, 1990). Worms were differentiated with respect to species and developmental stage, and counted in 20 ml aliquots of the wash/digest with a minimum sensitivity of 1–100.

Vomb

This trial was part of a large 2-year epidemiological study of abomasal nematodes of sheep in Sweden. The experiment was set up on a commercial sheep farm outside Vomb in southern Sweden, which had previously reported problems with nematode parasites and the presence of *H. contortus* in the flock.

The experiment was carried out on two naturally contaminated 2 ha pastures which had been used routinely each year for the grazing of sheep. During the summer of 2002, 20 ewes and their 28 lambs grazed on each of these two pastures. The accumulation of parasites was carefully monitored using faecal egg counts and tracer lambs each month (Waller *et al.*, 2004). The following year, four worm free tracer-lambs (born 2002) were released onto each paddock on 13 May 2003. The tracer test proceeded for three weeks after which tracers were slaughtered. The small intestine and abomasum were collected at slaughter and processed for worm recovery as previously described for Bergshamra.

Results

Development from eggs to larvae

No significant differences in the development of eggs to infective larvae were observed between the Swedish and Kenyan isolates (fig. 1a). In addition, there were no significant differences in the number of harvested larvae at the different time points for the faeces incubated at 15°C. However, at 5°C and −1 to 15°C no development of eggs occurred, although it was found that a small proportion developed into infective larvae when incubated at 25°C for an extra week. At 15°C, 9.5% and 12.3% of eggs of Kenyan and Swedish isolates, respectively developed into infective larvae. After the additional incubation these samples had a developmental rate of 36.4% of eggs for both isolates.

Long-time survival of infective larvae

No significant differences in the rate of survival were observed between the two isolates regardless of temperature. At all three temperatures, a substantial change in survival rate occurred between harvesting from culture (week 0) and the first sampling occasion (week 4). All data are shown in fig. 1b. After the first 4 weeks no major differences in survival were observed between the two isolates throughout the experiment, with the exception of the last sampling occasion for larvae stored at 15°C. From weeks 16 to 24, the rate of survival dropped from 66% to 29% in the Kenyan isolate and from 78% to 13% in the Swedish isolate.

Tracer studies

Malmö and Lövsta

Egg counts were zero for all animals at turnout in both years. In 2001 all animals were confirmed positive for *H. contortus* 7 weeks post-infection.

Faecal egg counts from 2002 tracer lambs showed that all four paddocks were contaminated with nematode parasites. All lambs had positive egg counts that ranged between 50 and 1100 epg. However, at autopsy adult *H. contortus* was found in only four of six tracer-lambs and in three ewes or from three of four plots (fig. 2a). No *H. contortus* were recorded on the plot infected with Kenyan worms in central Sweden, e.g. in Lövsta. No differences in the survival capacity were noticed, either when worm counts between the geographical locations, or between isolates, were compared. The number of recovered *Teladorsagia circumcincta* was higher than that for *H. contortus*, but both species were still found at very low levels (fig. 2a).

Bergshamra

All animals had a zero egg count at turnout. After 4 weeks all lambs had nematode faecal egg counts ranging between 250 and 900 epg, increasing to 350 to 1400 epg at the time of slaughter.

Worm intensities at the end of the experiment are shown in fig. 2b. None of the lambs had acquired *H. contortus*, however all were infected with *T. circumcincta* (range 2100–7600) with inhibition ranging from 11–42%.

Vomb

The group of tracers slaughtered in October 2002 had means of 55,450 *H. contortus* (100% inhibited) and 79,000 *T. circumcincta* (92% inhibited), respectively (fig. 2c).

Results from the tracer test performed in 2003 showed that *H. contortus* was present in only one of eight tracer lambs as inhibited larvae (mean 625). In contrast, extremely high levels (mean of 157,873) of *T. circumcincta* were found. Unlike the results from October the previous year, the proportion of inhibited *T. circumcincta* was much lower in the tracer test conducted in May 2003, ranging between 59% and 67%. All results from Vomb are shown in fig. 2c.

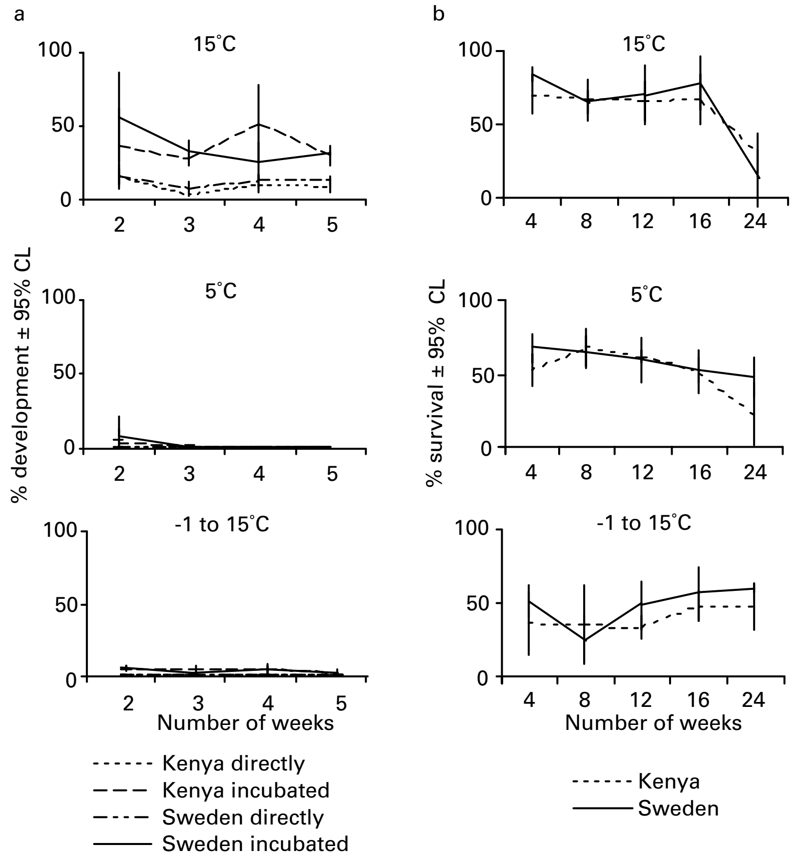

Fig. 1. The effect of temperature on the development and longevity of *Haemonchus contortus* eggs and infective larvae of Kenyan and Swedish isolates of *H. contortus*. a) Numbers of harvested infective larvae recorded (presented as percentage of theoretically possible development ± 95% confidence limits) when faeces were incubated at 5°C, 15°C or −1 to 15°C. Cultures were either harvested directly or after incubation at 25°C for an additional week. b) Living larvae recorded (± 95% confidence limits) when maintained at 5°C, 15°C or −1 to 15°C.

Meteorology

Mean weekly temperatures recorded throughout the experimental periods follow the long-time average temperatures at the different locations (fig. 3). As expected, the minimum temperatures are lower in central Sweden compared to those from the southern experimental sites (Vomb and Malmö).

Discussion

In the present study, no differences in development and survival were found between the two isolates of *H. contortus*, representing one temperate and one tropical example of this nematode species. In addition, it was shown that overwintering on pasture was of no practical significance in the transmission of *H. contortus* between grazing-seasons in Sweden. Thus, no evidence was provided for an adaptation to a northern temperate climate of the pre-parasitic stages of *H. contortus*.

As in previous studies, the development was most successful at the highest temperature for both isolates (15°C with following incubation at 25°C) (Silverman & Campbell, 1959; Crofton, 1965; Rossanigo & Gruner, 1995). Furthermore, little development took place from the second week onwards in samples maintained at 5°C and −1 to 15°C, although they were later incubated at 25°C. This indicates that the eggs and pre-infective larval stages died at the lowest temperatures tested. This is in agreement with Silverman & Campbell (1959), who showed that unembryonated eggs of *H. contortus* only survived at temperatures below 1.1°C for 3–7 days and no development took place below 9°C.

In contrast to these results, the long-time survival of infective larvae occurs at lower temperatures. Towards the end of the experiment, e.g. week 24, a reduced survival rate of the larvae, especially at 15°C, was observed. Similar studies on larval survival have been made on the closely related nematodes *Trichostrongylus colubriformis* and *Hyostrongylus rubidus* (Rose & Small, 1982, 1984) whereby survival rates dropped dramatically between weeks 16 and 24 at 22°C and 27°C, respectively. Larvae utilize lipids as their primary energy source and the dramatic change in survival rate after 16 weeks is likely to be due to the exhaustion of food reserves.

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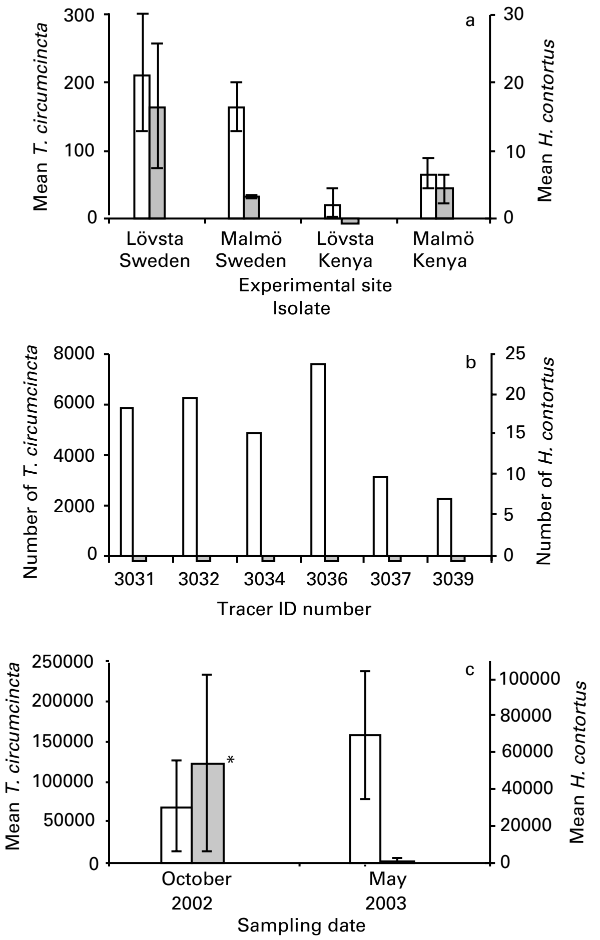

Fig. 2. a) Mean worm counts of *Haemonchus contortus* (■) and *Teladorsagia circumcincta* (□) from Malmö and Lövsta. b) Total worm counts from Bergshamra presented for each tracer animal. All tracers were male lambs. c) Mean worm counts from Vomb in October 2002 and May 2003. *Entirely EL4.

The present study also provides new information on the survival of free-living stages of *H. contortus* under Swedish winter conditions. Three tracer lamb experiments were conducted over two consecutive grazing seasons to highlight climatic influences on the level of overwintering survival. Although these experiments confirm the current opinion that *H. contortus* does not survive well on pasture under Swedish winter conditions, low infections were accordingly recovered.

In the Malmö and Lövsta trials, the overwinter survival of larvae with contrasting geographical origins was investigated. Experimentally infected sheep contaminated the pasture plots during one season and tracers were placed on the same plots the following year to monitor the presence of overwintered larvae. In this experiment, the degree of contamination of pastures during 2001 was influenced by the low stocking rate. In addition, at the time of slaughter, localized areas of the pasture plots in Malmö were covered by long grass, which led to a dilution effect of the infective larvae in the herbage. Extremely low infections of *H. contortus* (mean

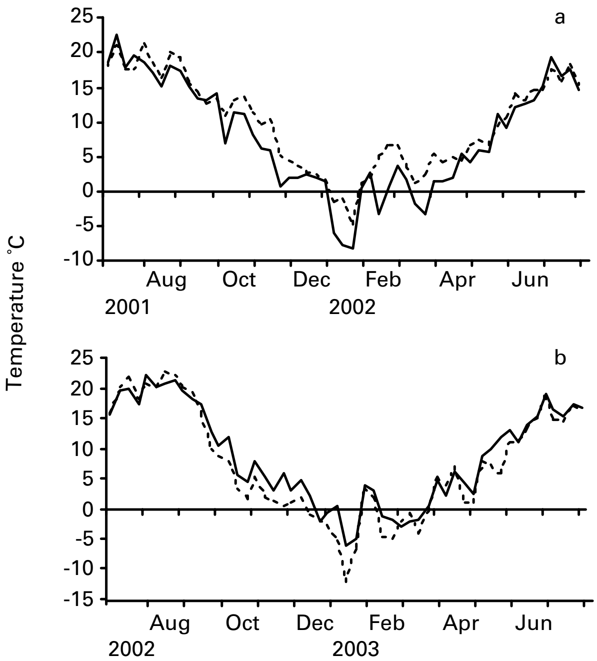

Fig. 3. Mean weekly temperatures from: a) the two experimental sites in Lövsta (———) and Malmö (– – –) used in the tracer trial during 2001 and 2002 and b) the two experimental sites in Vomb (———) and Bergshamra (– – –) used in the tracer trial during 2002 and 2003.

<10 adult worms) were acquired by the tracers that grazed for an extended period (6 weeks) on pasture following turnout.

However, the epidemiological consequences of this pickup are considered as irrelevant in relation to the data from the Bergshamra and Vomb studies, where the test for overwintering survival was conducted according to actual sheep management practices in Sweden. In the latter two studies, overwintered survival was tested at the time of turn-out using six and eight tracer lambs respectively, on pasture which the previous year had sheep grazing continuously for the whole season and at stocking rates that were appropriate for the size of the area and the available pasture quality.

In Bergshamra, there was no *H. contortus* acquired from the pastures, which the previous year had lambs dying of haemochiasis. Although there were low levels of *H. contortus* recorded in the Vomb tracers it should be noted that: (i) these were entirely inhibited in development; and (ii) these low numbers were observed when the same animals had burdens of *T. circumcincta* exceeding 150,000 worms, of which the majority were arrested in development. Although there are distinct morphological differences between early L4 *H. contortus* and *T. circumcincta*, when working with a background of one species being more than 100 times more abundant, then the chance of misidentification cannot be ruled out. On3 the other hand, as *T. circumcincta* was abundant it could be argued that low densities of *H. contortus* were overlooked.

Control strategies directed against *H. contortus* in Sweden are primarily being designed to interrupt transmission at critical points in the life cycle. Strategies

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currently being used in Sweden have been formulated on the assumption that there is no seasonal transmission of the parasite through free-living larvae overwintering on pasture (Rudby-Martin, 2002). Results from the three tracer-lamb studies conclude that contaminated pastures can support low numbers of *H. contortus* larvae at turnout the following year. However, the majority of worms picked up remained in the fourth larval stage of development. Inhibited populations of *H. contortus* at this time of the year are of no importance in perpetuating the nematode for the forthcoming season (Waller *et al.*, 2004). Thus the results indicate that overwintering of larvae on pasture in Sweden is of no practical significance. The high occurrence of arrested larval development (fig. 2c), indicates that this is the key to the epidemiology of *H. contortus* in Sweden.

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