Genotypic and Phenotypic Characterization of *Haemonchus contortus* in Sweden

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Abstract

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Haemonchus contortus is a blood-sucking nematode of the abomasum in small ruminants. It is responsible for extensive losses and huge animal welfare problems globally. This thesis is based on four publications with the overall aim to characterize *H. contortus* from Sweden, and includes both genotypic and phenotypic aspects of this parasite.

This nematode has by tradition been considered a tropical parasite due to the climatic requirements for its preparasitic lifestages. Its development and survival is dependent on temperature and humidity, and the window of opportunity to complete its life cycle in Sweden is limited.

The species status of *Haemonchus* in Sweden was investigated by comparing the genetic differences in the internal transcribed spacers (ITS-1 and ITS-2) of the ribosomal DNA (rDNA) in worms from Sweden and Kenya. As no fixed differences were found between these isolates they are considered to be distinct populations of the same species, *H. contortus*. The population structure of *H. contortus* at a global level was studied by analysing the genetic variability using both amplified fragment length polymorphism (AFLP) and *nad4* sequences of the mitochondrial genome. The results show that both the genetic variation and the genetic structure were high between the different isolates, and that populations from each continent mostly formed monophyletic groups in the phylogenetic analysis.

The thesis also includes experimental infection studies performed with both fresh and cold-treated infective larvae of either Kenyan or Swedish origin. These investigations covered a range of phenotypic traits, to search for any potential adaptations the parasite might have in order to survive Sweden's cold temperate climate. In addition, several studies were performed on the development and long-term survival of the parasite in climatic chambers, as well as studies on the overwinter survival of *H. contortus* on pastures in Sweden. The results show that this parasite can survive winters on pastures. However, the survival capacity was very low and is of limited significance in the transmission between grazing-seasons.

Taken together, the results presented in this thesis indicate that *H. contortus* has a large phenotypic plasticity rather than having undergone any evolutionary adaptation.

Keywords: Haemonchus contortus, Sweden, adaptation, phenotypic plasticity, acclimation, overwinter survival, parasitic nematode, sheep

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Svensk sammanfattning

Haemonchus contortus (Nematoda: Trichostrongyloidea) är en parasitisk rundmask som sprids via larver på betet till får och getter och drabbar framför allt ungdjur. De vuxna maskarna lever i värddjurets löpmage och livnär sig på att suga blod. *Haemonchus* har en direkt livscykel, vilket innebär att larverna som kläcks ur ägg på betet, äts upp tillsammans med betesgräset utan att parasiten behöver passera någon mellanvärd. Infektionens svårighetsgrad är beroende av hur många parasiter värden bär på. I värsta fall leder maskangreppen till att värddjuret dör av blodbrist, men vanligare är att man ser djur utan kliniska symtom men med försämrad tillväxt. Att kontrollera *H. contortus* är viktigt eftersom parasiten orsakar lidanden för djuren och leder till stora ekonomiska produktionsförluster.

Parasitens utveckling från ägg till infektiva larver är beroende av temperatur och fuktighet. Det har visat sig att varmt och fuktigt klimat är särskilt gynnsamt för parasitens utveckling och överlevnad. Problem till följd av *H. contortus* är följaktligen störst i tropiska och subtropiska regioner, men parasiten orsakar sjukdom även i tempererade klimatområden. I Sverige har *H. contortus* påträffats ända upp till polcirkeln. De klimatförhållanden som masken utsätts för i tempererade respektive tropiska områden väckte två frågor A) huruvida det är samma art som förekommer globalt och B) hur parasiten har anpassat sig till sådana skiftande miljöer.

Avhandlingen baseras på fyra delarbeten och inkluderar en studie om parasitens arttillhörighet, en populationsgenetisk studie samt experimentella infektionsstudier. De sistnämnda belyser olika fenotypiska karaktärer hos *H contortus* och genomfördes på lamm under stall- och fältförhållanden. Syftet var att få bättre kunskap om denna parasit och dess anpassning till det tempererade klimatet i Sverige.

Som en första del i arbetet undersöktes släktskapet mellan maskar från Kenya och maskar från Sverige för att fastställa arttillhörigheten för *Haemonchus* sp. hos får och get. Detta undersöktes genom att påvisa eventuella genetiska skillnader i det ribosomala DNAt. Mellan de kodande generna i det ribosomala DNAt finns sekvenser som visat sig variera mellan närbesläktade rundmaskar. Dessa sekvenser (ITS-1 och ITS-2) är de genetiska regioner som tidigare används för molekylära släktskapsstudier av parasitära maskar. Denna studie visade, att maskar från Kenya och Sverige är av samma art, dvs. inga skillnader påvisades i ITS-1 och ITS-2, men att isolaten representerar olika populationer.

Tidigare studier har visat att *H. contortus* har en populationsstruktur som tyder på ett genflöde mellan populationer. Det har också visats att maskisolat som passeras i värddjur under experimentella förhållanden kan förlora sin genetiska variation. Detta skulle kunna innebära att representativiteten hos våra laboratorieisolat kan ifrågasättas. För att undersöka detta genomfördes en populationsgenetisk studie med olika maskisolat från sex kontinenter. De metoder som används var A) sekvensering av en mitokondriell gen, *nad4,* och B) AFLP.

Resultaten visade att *H. contortus* uppvisar en hög grad av populationsgenetisk struktur såväl på global nivå som inom Sverige. Studien visade också att den genetiska variationen i det kenyanska och det svenska isolatet inte hade minskat i förhållande till övriga populationer från respektive kontinent och att de grupperade med dessa i den fylogenetiska analysen. Detta visar att dessa isolat fortfarande är representativa för sina respektive ursprung.

Studier med parasitens externa livsstadier i klimatkammare genomfördes i syfte att studera om *H. contortus* genomgått en anpassning till kallare klimat. För detta jämfördes åter de två laboratorieisolaten från Kenya och Sverige. Parasitäggens överlevnads- och utvecklingsförmåga studerades vid olika temperaturer. Även de infektiva larvernas tolerans för olika temperaturer jämfördes. Resultat från dessa studier visar att *H. contortus* från Sverige inte utvecklat någon unik förmåga att överleva och utvecklas under nordiska klimatförhållanden.

Bevis för genetisk anpassning till ett tempererat klimat uteblev när maskens infektionsförmåga studerades. I denna studie inokulerades lamm med infektiva larver av antingen det kenyanska eller svenska isolatet av *H. contortus* varefter olika fenotypiska karaktärer undersöktes. Två separata försök genomfördes: A) i det ena försöket användes färska larver, och B) i det andra försöket användes larver som förvarats vid 5 ºC i nio månader. Resultaten visade att larverna var infektiva trots att de varit utsatta för köldstress, men att prepatensperioden, förmåga att gå i vilofas och andelen larver som etablerades påverkades av föregående larvhantering. Små skillnader observerades mellan de studerade isolaten i respektive försök.

Inom ramen för denna avhandling har fyra betesförsök genomförts för att studera *H. contortus'* övervintringsförmåga på beten i Sverige. Resultaten från dessa försök visar att parasiten kan överleva den nordiska vintern. Andelen överlevande larver var dock extremt låg och den epidemiologiska betydelsen av smitta från föregående betessäsong kan ifrågasättas. Klart är att övervintring inuti värddjuret som hypobiotisk larv är parasitens huvudsakliga strategi för att överleva vintern i tempererade områden.

Denna avhandling sammanfattar resultaten från både en taxonomisk och en populationsgenetisk studie med molekylära metoder samt studier av klimateffekter genom fält- och infektionsförsök. Resultaten visar att *Haemonchus* i Sverige tillhör arten *H. contortus*, det vill säga samma art som infekterar små idisslare världen över. Studierna på parasitens fenotypiska egenskaper visar att *H. contortus* inte genomgått någon dramatisk evolutionär anpassning för att klara det för arten ogynnsamma klimat som är rådande i Sverige.

Contents

Appendix

Papers I-IV

The present thesis is based on the following papers, which will be referred to by their roman numerals.

I. Troell, K., Mattsson, J.G., Alderborn, A. & Höglund, J. 2003. Pyrosequencing™ analysis identifies discrete populations of *Haemonchus contortus* from small ruminants. International journal for parasitology 33, 765-771.

II. Troell, K., Engström, A., Morrison, D.A., Mattsson, J.G. & Höglund, J. 2006. Global patterns reveal strong population structure in *Haemonchus contortus*, a nematode parasite of domesticated ruminants (submitted).

III. Troell, K., Tingstedt, C. & Höglund, J. 2006. Phenotypic characterization of *Haemonchus contortus*: a study of isolates from Sweden and Kenya in experimentally infected sheep. Parasitology, 132, 403-409.

IV. Troell, K., Waller, P. & Höglund, J. 2005. The development and overwintering survival of free-living larvae of *Haemonchus contortus* in Sweden. Journal of helminthology 79, 373-379.

Abbreviations

AFLP	amplified fragment length polymorphism
AR	anthelmintic resistance
ATP	adenosine triphosphate
bp	base pair
BZ	benzimidazole
CCD	charge coupled device
DNA	deoxyribonucleic acid
EHT	egg hatch test
EL4	early fourth stage trichostrongyle larva / inhibited larvae
Epg	eggs per gram faeces
FECRT	faecal egg count reduction test
ITS	internal transcribed spacer
KRAV	The Swedish certification body for organic production
L3	infective third stage trichostrongyle larva
LSU	large subunit
LTA	long term average
mtDNA	mitochondrial DNA
PCR	polymerase chain reaction
PGS	population genetic structure
PPi	pyrophosphate
RAPD	randomly amplified polymorphic DNA
rDNA	the DNA coding for a ribosomal gene
RFLP	random fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal RNA
SLU	Sveriges lantbruksuniversitet (Swedish University of Agricultural Sciences)
SNP	single nucleotide polymorphism
SSU	small subunit
SVA	Statens veterinärmedicinska anstalt (National Veterinary Institute)
SWEPAR	Department of parasitology, SLU / SVA

Objectives of the present work

The main objective of my work was to gain knowledge of the parasitic nematode *Haemonchus contortus* from a Swedish perspective and to test a number of hypotheses regarding phenotypic and genotypic characters.

More specific, the aims of the study were as follows:

- Study the genetic relationship between *Haemonchus* of small ruminants from Sweden and Kenya, to investigate the species status of *Haemonchus* in Sweden.

- Investigate the population genetic structure of *H. contortus* at a global level.

- Compare different genetic markers and their usefulness in population genetic studies of *H. contortus.*

- Characterize *H. contortus* in Sweden with respect to a range of phenotypic characters.

- Study if *H. contortus* has adapted to a cold temperate climate and changed its life-history strategies accordingly, such as overwinter survival and inhibition pattern.

In order to examine the first aim, I compared genetic differences in the ribosomal DNA of three populations of *Haemonchus*, as discussed in paper I. The second and third aims are covered in paper II, which is a population genetic study of populations representing *H. contortus* from all continents. Finally, the fourth and fifth aims are covered in papers III and IV, where I assess the overwinter survival as well as characters such as worm establishment, prepatent period, inhibition, development of eggs and survival of infective larvae.

Background

Haemonchus contortus is a highly pathogenic nematode parasite and does, unlike other gastrointestinal helminth parasites in small ruminants, cause acute disease and occasionally even death. In recent years the Swedish National Veterinary Institute has received an increasing number of cases of sheep and lambs that have died from haemonchosis.

When something out of the ordinary is observed it usually awakens the curiosity of a researcher to ask the question why, and try to find the answer. In this thesis the question 'what is *Haemonchus contortus* of Sweden, and what is it doing here?' was asked. The question arose since this nematode by tradition has been considered a tropical parasite, and its climatic requirements are thus far from what Sweden has to offer. *H. contortus* has been present in Sweden for at least a few decades (Nilsson, 1973), but it is even more recently that *H. contortus* has been considered a problem and even a threat to the Swedish sheep farming. Why is it here and how does it survive and complete its life cycle? Is it an acclimation, a result of phenotypic plasticity, or genetic adaptation as a result of an evolutionary process? Is *H. contortus* in Sweden even the same species as its congeners in the tropics?

Haemonchus contortus

Basic characteristics

The stomach- or wireworm, *H. contortus,* was first described in 1803 by Karl Rudolphi (Soulsby, 1982). This blood-sucking abomasal nematode parasite of sheep and goats belongs to the superfamily Trichostrongyloidea (Urquhart *et al.*, 1996), and is the single most important nematode pathogen of small ruminants in the developing world (Perry *et al.*, 2002). There are 12 species of *Haemonchus* described, of which *H. contortus*, *H. placei* and *H. similis* are globally distributed in the abomasums of domesticated ungulates within the subfamilies Caprinae and Bovinae (Hoberg, Lichtenfels & Gibbons, 2004).

The infection causes anaemia and occasional death of the animals, and is a major animal welfare problem. The main problem for the farmer is production losses due to decrease in weight and growth of the host animal that in turn leads to economic losses. *H. contortus* is frequently found in tropical and subtropical regions, where the conditions for the survival of this parasite are optimal. However, the parasite has also become a growing problem in temperate regions (Waller *et al.*, 2004b).

Life cycle and climatic limitations

Haemonchus has a direct life cycle, and is transmitted horizontally on pasture by third-stage larvae (L3). The life cycle is divided into a free-living phase, and a parasitic phase inside the host (Fig. 1). Eggs are passed in faeces, and develop

through the first (L1) and second larval (L2) stages to the infective L3. *H. contortus* is ingested with herbage and then travels to the predilection site in the abomasum, where the larva moult twice and develop into the sexually mature adult stage. The prepatent period is approximately 2-3 weeks (Thamsborg, Søland & Vigh-Larsen, 2001; Urquhart *et al.*, 1996).

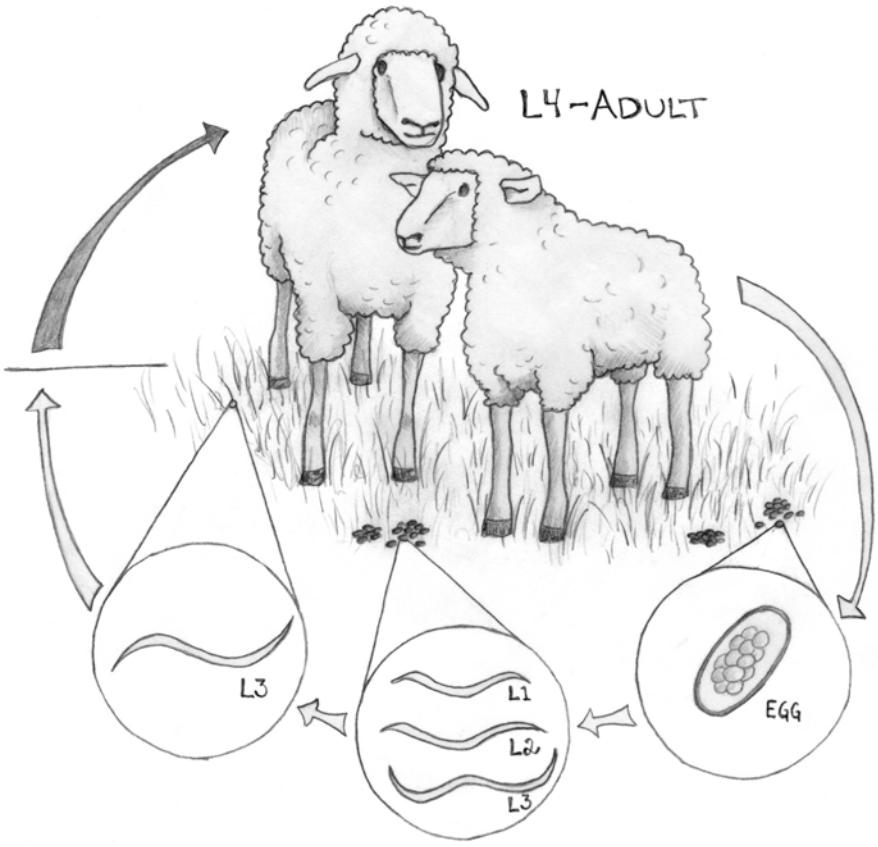

Fig. 1. The life cycle of *Haemonchus contortus*. For a description of the life cycle see the text. The darker arrow represents the parasitic phase of the life cycle.

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 the external stages of *H. contortus*, from eggs to L3. The lower environmental limits for haemonchosis in sheep have been identified as a mean monthly temperature of 18 °C and a minimum monthly rainfall of 50 mm (Gordon, 1948). The optimal condition for development from egg to L3 is 28 °C with humidity greater than 70% (Rossanigo & Gruner, 1995), and little or no development of eggs to larvae will take place

below 9 °C (Silverman & Campbell, 1959). On this basis, the environmental conditions in which external larval stages of *H. contortus* can develop and complete their life cycle are limited in cold temperate climates.

Inhibited development, arrested development or hypobiosis is an important aspect of the biology of gastrointestinal nematodes of ruminants (Eysker & Kooyman, 1993). Seasonal change has been suggested as a major cause of inhibition for *H. contortus* also (Connan, 1971; Muller, 1968). Accordingly, this phenomenon is believed to be an adaptation to survive the winters in temperate regions, when the opportunities for larval transmission are restricted (Blitz & Gibbs, 1972; Waller & Thomas, 1975). Although summer inhibition occurs to avoid the dry season (Eysker & Kooyman, 1993), hypobiosis in tropical and subtropical areas is less commonly reported (Allonby & Urquhart, 1975). However, others suggest that inhibition in *H. contortus* is an obligatory genetic strategy for survival rather than an effect of external stimuli (Waller & Thomas, 1975).

H. contortus in Sweden

H. contortus was widespread in Sweden, especially in the South, until the 1960's (Rudby-Martin & Nilsson, 1991). However, even though the parasite was present throughout Sweden it seldom caused severe haemonchosis. In the 60's, strategic programs of deworming with benzimidazoles (BZ) reduced the prevalence, resulting in nearly two decades free of *Haemonchus* problems.

The first report of resistance to BZ in *H. contortus* was in 1991 (Rudby-Martin & Nilsson, 1991). Once again, *H. contortus* spread to become a problem, and in 1992 65% of farms in the southern Sweden had *Haemonchus* in their flock, and 3% for the rest of the country (Nilsson *et al.*, 1993). In a more recent study of the prevalence of *H. contortus* in organically raised sheep in Sweden, 37% of the flocks examined were infected, and the northernmost finding was close to the Arctic Circle (Lindqvist *et al.*, 2001).

Recently it was demonstrated that *H. contortus* in Sweden has developed some unique epidemiological adaptations. Most important are both the high propensity to arrest development and the onset being very early in the season (Waller *et al.*, 2004b). High levels of arrested development of Swedish *H. contortus* have been reported also in experimentally infected lambs (Hrabok *et al.*, 2006; Waller *et al.*, 2004a).

It has been suggested, both in Sweden and Norway, that *H. contortus* has limited survival on pastures over winter (Helle, 1971; Waller *et al.*, 2004b; Waller *et al.*, 2006). The key to the transmission of this parasite between seasons has been suggested to be inhibition of larvae (Waller *et al.*, 2004b). Thus, under Swedish sheep farming conditions *H. contortus* survive the long cold winters within the host as arrested larvae, and the parasite relies on the lambing ewe to complete its life cycle. This is of practical importance as, if the majority of the parasite population is overwintering inside the host, this creates the opportunity for

eradication. Preliminary results also suggest that, with proper anthelmintic treatment of all animals during the housing period, *H. contortus* can be eradicated on a farm-by-farm basis in Sweden (Waller *et al.*, 2006).

It has been suggested that interchange between sheep and cattle grazing could transmit the parasite between the two host species. Although previous studies have shown that *H. contortus* also can cycle in young cattle (Achi *et al.*, 2003; Amarante *et al.*, 1997; McGhee *et al.*, 1981), there are, to my knowledge, no such confirmed findings reported in Sweden. The only recent finding of *Haemonchus* sp. in Swedish cattle where identification to species has been performed was in 2001. The identification was performed with molecular markers in ITS-2 (Stevenson, Chilton & Gasser, 1995), and the results clearly showed that the isolated worm was *H. placei* (Troell, unpublished data).

It has also been suggested that wild ruminants may act as reservoirs. Wild cervids, such as roe deer and moose, are common in Sweden (Divina *et al.*, 2000). The epizootiological potential of these hosts as reservoirs, and whether reciprocal infections between wild and domestic animals can occur, has been studied. The outcome of necroscopy of Swedish roe deer showed that a few specimens of *H. contortus* were found in one animal (0.2%) of 462 investigated abomasa (Nilsson, 1971). There is also a record of *H. contortus* in moose in Sweden (Nilsson, 1971). Another potential host in northern Scandinavia that can transmit ovine nematode parasites is reindeer, which are commonly raised in northern Sweden. In a study performed with experimentally infected reindeer, it was shown that reindeer are susceptible to *H. contortus* (Hrabok *et al.*, 2006). However, it is during the winter months, December to February, that reindeer are kept on the same fenced-in areas where sheep are kept during the spring and summer (Hrabok *et al.*, 2006), when development and survival of *H. contortus* external stages are very unlikely to occur.

Climate of Sweden

Sweden’s climate varies from north to south (Fig. 2), temperate in the south with cold cloudy winters, and cool partly cloudy summers and sub-arctic in the north. In general, the climate can be considered temperate, due to the offshore Gulf Stream waters. Two important factors influence Sweden's climate: its northern latitude (between 55 and 69 °N) and the shelter from milder and wetter Atlantic winds by the mountains along its western border with Norway. The summers are warm, but get cooler further north. Winters can be bitterly cold, especially in the north where days are shorter and heavy frosts and bountiful snowfalls are common.

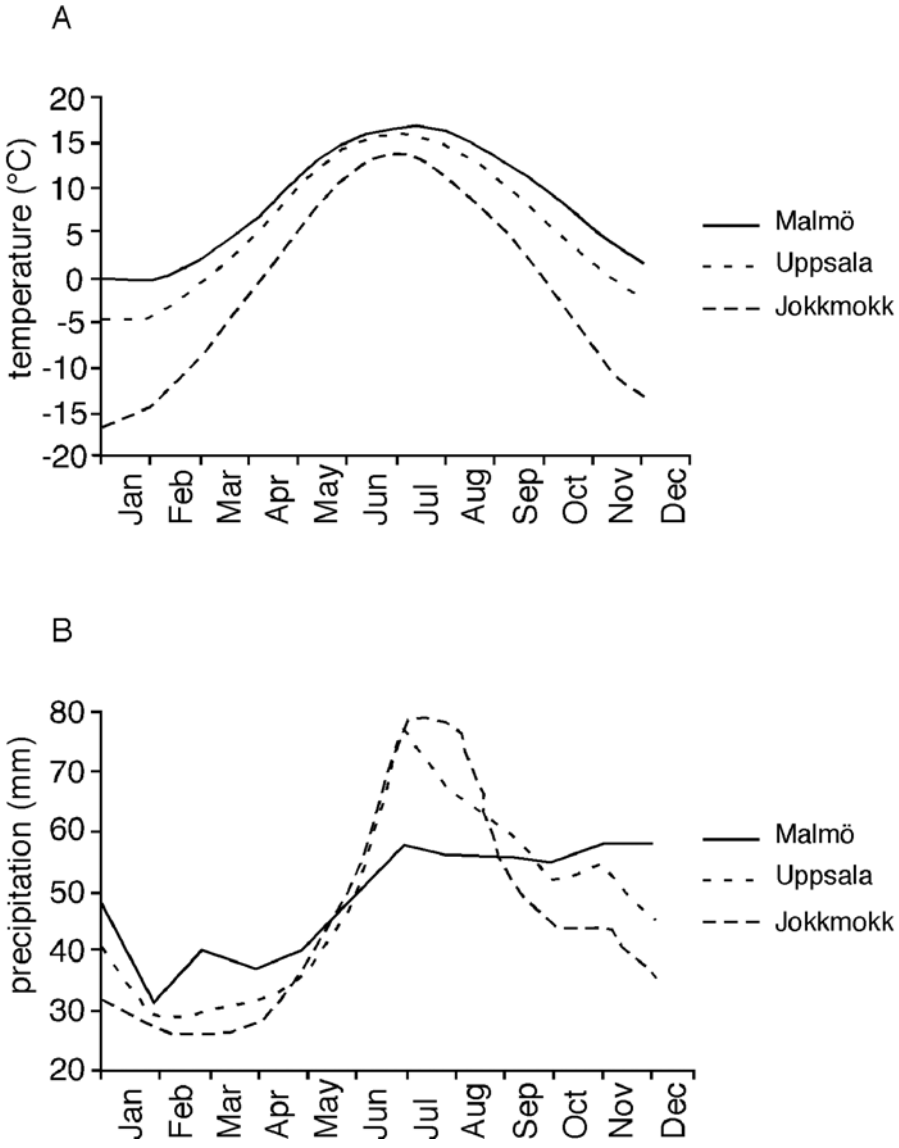

Fig. 2. Meteorological data representing South, Central and Northern Sweden, respectively. The data shown are the long term (30-years) average (LTA). A) LTA monthly mean temperature. B) LTA monthly mean precipitation. The data are collected in Malmö (lat. 55.34, long. 13.05), Uppsala (lat. 59.52, long. 17.38), and Jokkmokk (lat. 66.36, long. 19.49). Jokkmokk is located north of the Arctic Circle (lat. 66.33).

Sheep and goat farming in Sweden

Approximately 8 200 sheep flocks, with a total of 220 000 winterfed ewes and rams and 246 000 lamb, are registered in Sweden (these figures refer to holdings of more than 2 hectares of land) (Jordbruksverket & Statistiska centralbyrån, 2005). Swedish sheep farming is increasing, and there were 8% more sheepholders registered in 2004 than in 2003. The average flock (excluding lambs) in Sweden is 27 sheep per farm (Jordbruksverket & Statistiska centralbyrån, 2005). The density of sheep production is mainly concentrated in the south and on the island of Gotland, although there is sheep production throughout Sweden (Lindqvist *et al.*, 2001; Nilsson, 1973). Swedish sheep stock mainly consists of animals originating from older native breeds. However, it is also common to have crossbreeds from ewes with high fertility (Finewool) and heavy meet breeds (such as Texel). The Gotland (pälsfår) is the predominant sheep breed, which is a native breed that, since the early 1920's, been created through a steady selection for fur quality (http://www.sjv.se/home/amnesomraden/animalhealthwelfare/swedishbreedsofdomesticanimals/sheep; 20-Mar-2006).

In addition, Sweden has about 500 flocks of goats, with a total of 5 500 animals. About 20% of the goats in Sweden are raised in the province of Jämtland (Jordbruksverket & Statistiska centralbyrån, 2005).

There is a trend towards an increase in organic agricultural production in Sweden. This increase is supported by a governmental policy with the stated aim that 10% of all lamb meat produced in Sweden should be from organically reared animals by 2010 (Jordbruksverket, 2004). There are no official figures on how many organic farms hold sheep or goats, but there should be many more than the KRAV-certified flocks (http://leine.no/vet/gs/forebygg/adferd/eko_2.htm; 17-Jan-2006). The number of sheep and goat flocks that were registered in 2004 to rear organically according to the standards of KRAV were 512 and 10, respectively (http://statistik.krav.se/rPortfolio/generate/PageViewer.asp; 11-Apr-2006).

The Swedish lambing season is mainly during the spring months (March-May). The lambs are allowed to reach a live weight of approximately 40 kg, which is generally done within the same year. However, the lambs that fail to do so are kept indoors, and are slaughtered early the following spring (Lindqvist *et al.*, 2001; Nilsson, 1973). Due to the different climates in the north and south of Sweden, the grazing period varies depending on where in the country the sheep are raised. In the south the grazing period normally starts in early May and ends in October. In the north the grazing season is considerably shorter, starting in June and ending in September. Sheep are in general housed during the rest of the year, but on some farms it is also common to let the animals pass free into pens outside the house throughout the year (Nilsson, 1973).

Species delimitation

There are many definitions of what represents a species (Cracraft, 2000). The problem of deciding what species are, and how they should be delimited in nature, has been one of the most active areas of evolutionary biology (Turelli, Barton & Coyne, 2001). However, when two populations are defined as different species it is assumed that these two populations appear on independent evolutionary trajectories and that they will continue to be so in the future (Adams, 1998). There are several obstacles when using morphological, behavioral, physiological or biochemical characters to define a species, as selection pressure can lead to almost identical phenotypes of different organisms that are not related to each other (Kunz, 2002).

The attractions of using molecular (*i.e.* genetic) data for defining and separating species are compelling and nowadays usually unchallenged. Molecular data allow comparison without influence of factors such as life stage, age, host or geographic origin (Nolan & Cribb, 2005). In addition, methods based on the polymerase chain reaction (PCR) have found broad applicability, as its sensitivity permits the analysis of markers from limited amounts of DNA. This is particularly relevant in studies of single parasitic nematode individuals, as it is not possible to obtain large amounts of DNA material or to cultivate them *in vitro*.

For example, the genus *Haemonchus* is composed of >10 species, where *H. contortus* and *H. placei* are two of the important veterinary species (Hoberg, Lichtenfels & Gibbons, 2004). It has been suggested that these two are a single species, since there are only minor morphological differences between them, but that there has been strain adaptation for sheep and cattle (Gibbons, 1979; Urquhart *et al.*, 1996). However this issue was resolved by molecular methods by Stevenson, Chilton & Gasser (1995). By use of a marker in the ribosomal DNA (rDNA) they could conclude that these were indeed two different species.

Molecular markers

During the last few decades remarkable progress has been made in the field of molecular genetics. This has led to the development of a variety of different techniques that can be used to study genetic variation. All marker systems detect variation in one of three different classes of marker: protein variation (*e.g.* allozymes), DNA sequence polymorphism (*e.g.* DNA sequencing, SNP, RFLP, AFLP etc.) and DNA repeat variation (*e.g.* minisatellites, microsatellites) (Schlötterer, 2004). These genetic markers may differ with respect to important features, such as abundance, level of polymorphism detected, locus specificity, reproducibility, as well as technical requirements and financial commitments (Woodhead *et al.*, 2005). Therefore, the usefulness of a marker depends on its characteristics, and the choice of marker must be based on the specific application. Hence, no marker is generally superior to all others. An ideal marker would have sufficient variation for the problem under study, be reliable, and be simple to generate and interpret. Sufficient variation for species delimitation should be

relatively low intraspecific variation and a high level of divergence between closely related taxa (Vilas, Criscione & Blouin, 2005).

The markers used for papers I and II will be discussed in detail below.

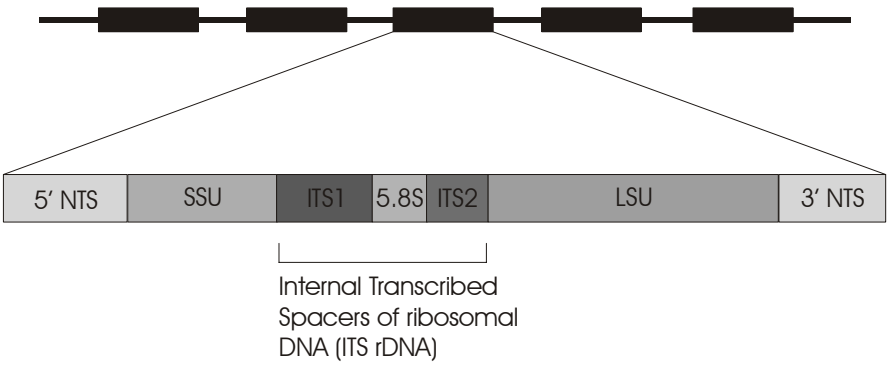

Fig. 3. Arrangement of ribosomal DNA. The tandem repeats are drawn as black boxes. The non-transcribed spacers (NTS) and the transcription unit including the small subunit (SSU), the large subunit (LSU), and the 5.8S genes and two internal transcribed spacers (ITS1 and ITS2) (figure not to scale).

Nuclear genes, rDNA, Internal Transcribed Spacers

The ribosomal DNA is a multigene family, with nuclear copies arranged in tandem arrays in most species. In these arrays the internal transcribed spacers (ITS) is located between the small subunit (SSU) and the large subunit (LSU) rRNA genes, and is divided into two regions (ITS-1 and ITS-2) separated by the 5.8S RNA gene (Fig. 3) (Schlötterer *et al.*, 1994).

The small subunit gene is highly conserved and its sequence has been used to shed light on deep evolutionary branches. The molecule has accounted for enormous insight into evolutionary biology, and has proven to be an important marker in our understanding of the origin and diversity of species (Van de Peer *et al.*, 2000). The SSU is a popular species-specific marker in both prokaryotes and eukaryotes, and tens of thousands of complete or partially complete SSU sequences are available in public databases. The SSU has been proposed as one of the standard DNA barcoding genes (Blaxter, Elsworth & Daub, 2004).

Many studies in the molecular phylogenetic research of strongylid nematodes including the superfamily Trichostrongyloidea, have been based on nuclear rDNA sequence comparisons, in particular from the ITS (for reviews see Gasser *et al.*, 2004 and Gasser & Newton, 2000). Both the ITS-1 and ITS-2 can be amplified with universal primers targeting the conserved genes flanking the spacers (Heise, Epe & Schnieder, 1999). Sequence data of the ITS of the rDNA are faster

evolving than the SSU, and have therefore been extensively used and have proved to be a high-resolution marker for species identification for a wide range of closely related parasitic nematodes (Anderson, Blouin & Beech, 1998).

Ribosomal DNA genes usually evolve cohesively within a single species and exhibit only limited sequence divergence between rDNA copies within a single individual. This homogenisation of the rDNA is referred to as concerted evolution (Anderson, Blouin & Beech, 1998). However, in parasitic nematodes multiple sequences of ITS are frequently found within individual worms (Hoste *et al.*, 1995; Nadler *et al.*, 2000b; Troell *et al.*, 2003). Both direct sequencing and cloning of PCR products can be used for ITS analysis. Direct sequencing generates a consensus sequence that can be used for phylogenetic analysis. In contrast, questions about individual polymorphism can be answered with cloning and sequencing of PCR products.

Fragment analysis, AFLP

With amplified fragment length polymorphism (AFLP), DNA fingerprints are generated by selective amplification of restriction fragments. The major advantage of the AFLP technique is the large number of polymorphisms that the method detects; *i.e.* this marker is hypervariable and generates unique banding pattern for each individual. Other advantages are that it is efficient to use on individual worms, it covers the whole genome with no previous knowledge of the sequence required, and since the AFLP technique is PCR-based it can provide high throughput (Höglund *et al.*, 2004; Nejsum *et al.*, 2005; Otsen *et al.*, 2001).

AFLPs are generally acclaimed for their reproducibility, which makes the technique superior to the use of RAPDs. RAPD markers have been shown to be sensitive to small changes in the reaction conditions, and may express polymorphisms of nongenetic origin (Kjølner *et al.*, 2004; Perez, Albornoz & Dominguez, 1998). The reproducibility of AFLPs was tested throughout a network of European laboratories, and by rigorously control of all of the variables they were able to show that it is possible to reproduce AFLP banding patterns across a range of laboratories (Jones *et al.*, 1997).

The claims for AFLP reproducibility are well-founded, but there are several concerns to take into account: the method of genomic DNA preparation may affect banding patterns, *e.g.* partial digestion due to either poor DNA quality or insufficient restriction enzymes, and the scoring of AFLP fragments is open to a certain amount of interpretation, which suggests that different researchers may score AFLP patterns differently.

Homology as well as non-independence is perhaps the greatest problems in AFLP analysis. It is often assumed that co-migrating bands are homologous (Woodhead *et al.*, 2005). However, a particular-sized band may consist of several bands from different regions of the genome. In addition, there is no way to assess the homology of missing bands. On the other hand, when a nucleotide substitution creates a restriction site, three bands, all representing the same region in the

genome, will be created and scored separately, *i.e.* non-independence of bands. The loss of a restriction site is most likely caused by a point mutation in the restriction enzyme recognition sequence, causing the sequence not to be recognised by the enzyme and therefore not cut. Likewise the gain of a site is caused by a point mutation changing a potential site into a recognisable site. The probabilities of these two events are unequal, with the loss of a restriction site being the much more common event (DeBry & Slade, 1985). Site loss or gain may also be produced by an insertion, deletion or duplication event.

AFLP markers are dominant, with polymorphisms detected as either band presence or absence. Dominance means that one allele masks the presence of the other, the recessive allele, so that heterozygotes of genotype dominant/recessive (codominant) are not distinguishable from homozygotes of genotype dominant/dominant (Piepho & Koch, 2000). In contrast, a codominant marker allows unequivocal distinction of homozygous and heterozygous genotypes. Codominant markers are considered to be more efficient for population genetics studies, as considerably fewer individuals need to be sampled per locus than for dominant markers (Lynch & Milligan, 1994; Piepho & Koch, 2000). However, this may be overcome with use of AFLP, because of the large number of polymorphisms generated with this method (Krauss & Peakall, 1998). For example, in a direct comparison of AFLP and various codominant mitochondrial DNA (mtDNA) markers for the lungworm *Dictyocaulus viviparus*, there was qualitative agreement between both methods, but with more genetic variation obtained with AFLP. However, the mtDNA seemed to be more informative about the most recent history of the parasite populations (Höglund *et al.*, 2006).

Mitochondrial DNA, nad4

Each mitochondrion has its own genome, which is distinct from, but cooperates closely with, the nuclear genome. The analysis of mtDNA has proven to be a useful molecular marker for evolutionary studies in animal populations, and has become an important tool for studying population structure and history (Avise, 2004). This is because, in contrast to nuclear DNA, it predominantly follows clonal maternal inheritance patterns (with few exceptions *i.e*. heteroplasmy, mtDNA are inherited from one generation to the next through the maternal line) (William, Ballard & Rand, 2005), with no or little recombination, has relatively rapid base substitution rate, and is easy to isolate (Avise *et al.*, 1987). The assumed lack of recombination allows sequences to be traced through one genetic line, and all polymorphisms can be assumed to be caused by mutations.

In general, mtDNA has a higher rate of substitution than does nuclear DNA (Blouin, 2002), making it possible to resolve differences between closely related individuals. The fast substitution rate of animal mtDNA is, in part, due to inefficient mutation repair mechanisms (Avise, 2004). Rapidly evolving sequences may get saturated with substitutions, where multiple events have occurred at many sites. Saturation causes problems for higher-level phylogenies (Blouin *et al.*, 1998), and meaningful comparisons from mtDNA are therefore limited to closely related taxa or population genetic studies (Avise *et al.*, 1987). Different parts of

the mtDNA change at different rates, *e.g.* the control region very rapidly, the tRNA genes more slowly (Moritz, Dowling & Brown, 1987; Randi, 2000).

Most cells contain hundreds or thousands of mtDNA molecules (Randi, 2000). All of these copies per cell result in the need for less parasite material when using mtDNA marker compared to a nuclecar DNA marker. This is a great advantage as lack of material is a common problem, since helminth parasites cannot be grown *in vitro*. In particular, the *nad4* and *cox1* genes of the mtDNA have been used for population genetic studies of several parasitic nematodes (reviewed by Höglund *et al*., 2006).

Several characteristics suggest that the evolution of nematode mtDNA is unusual compared to other phyla. For example, nematode mtDNA tends to be extremely AT rich, with typical values between 75 and 80% (Anderson, Blouin & Beech, 1998; Blouin *et al.*, 1998). There is evidence that nematode mtDNA evolves fast (Anderson, Blouin & Beech, 1998; Blouin *et al.*, 1995; Blouin *et al.*, 1998), and that the mitochondrial genome of nematodes may be more prone to recombination and gene rearrangement than other organisms (Blouin *et al.*, 1998). The mitochondrial genomes of nematodes are usually smaller (~13.6 to 14.3 kb) than those of other metazoan groups (Hu, Chilton & Gasser, 2004). The known exceptions are *Trichinella spiralis* and *Romanomermis culicivorax*, which have mitochondrial genomes in size of ~21-26 kb (Hu, Chilton & Gasser, 2004).

Materials and methods

Further details about the materials and methods used are given in papers I-IV.

Worm isolates

For all of the studies presented in this thesis, two isolates have been used for comparative reasons: one isolate from Sweden representing a temperate climate origin, and one from Kenya representing a tropical climate origin.

Swedish isolate

The Swedish isolate originates from a farm in Noraström (latitude 63 °N), Västernorrland, in northern Sweden. The isolate was initially established in 2000 (Table 1). Approximately 200 adult *H. contortus* from experimentally infected sheep were identified at slaughter, and surgically transferred directly into the abomasum of a worm free recipient lamb. The resistance status of this isolate has not been formally tested. However, several preliminary results based on FECRT, sequencing of β-tubulin codon 200, and the egg hatch test (EHT), indicate that this isolate is resistant to BZ (unpublished data).

Kenyan isolate

The Kenyan strain was isolated on a farm on the Kapiti plains (latitude 1 ºS) in 1998, and has since then been maintained in experimentally infected sheep kept at the International Livestock Research Institute (ILRI) in Nairobi. Larvae from these sheep were sent to Sweden in September 2000, and experimentally established in Swedish sheep. This isolate has been tested for anthelmintic resistance (AR) and has proved in a FECRT to be highly susceptible to levamisole, albendazole and ivermectin (Githiori *et al.*, 2004). We have also performed several studies with preliminary results based on direct sequencing of the β-tubulin gene, codon 200, and EHT that support the documented susceptibility to BZ (unpublished data).

Additional isolates

Isolates used for paper II were collected as adult worms of both sexes, and preserved either by ourselves or by colleagues at other laboratories or abattoirs. After collection they were sent either fresh frozen or frozen in alcohol to SWEPAR. All worms were stored separately at –70 ºC until DNA extraction was performed. The worms represented 19 populations mainly from sheep, and were of both field and laboratory origin.

Culture and storage conditions

Both the Kenyan and the Swedish isolates have been passaged annually in sheep since 2000 in Sweden (Table 1). Each time, 2-4 helminthologically naïve young lambs were inoculated with 2000-3500 infective larvae. The infection was allowed to become patent, and faeces were then collected in faecal collection bags. The

faeces were collected on a daily basis for several weeks and cultured at room temperature for 10 days, and the L3s were then recovered with Baermann technique. Larvae were stored in cultures at 5 ºC or 10 ºC (after the first passage only). 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 or third passage that had been stored at 5 ºC (Table 1).

Table 1. Treatment and number of passages in the Swedish and Kenyan isolates (after establishment in Sweden).

Passage	Year	Storage	Larval age	Used for studies
Establishment	2000			Paper I (10 worms)
1	2001	10 °C	11 months	Paper I (additional 30 worms) Paper II Paper IV (Lövsta and Malmö)
2	2002	5 °C	6 months	Paper IV (L3 survival and egg development)
3	2003	5 °C	10 months	Paper III (cold-treated)
4	2003	5 °C	Fresh	Paper III (fresh)
5	2004	5 °C	12 months	
6	2005	5 °C	12 months	

Larval doses

For the artificial infections, each lamb was given a dose of 2000-3500 infective larvae. Before the preparation of the infection doses, larvae were first allowed to adjust to room temperature for 5-8 hours. The larvae were poured into a conical glass, and the larval suspension was aerated to get a homogenous solution. From this, larvae were counted in 5 sub-samples of 25 µl. Individual doses were prepared in 50 ml Falcon tubes. To ensure that cross contamination between the different isolates did not occur, this was always performed at different tomes.

For the stable trial (paper III) the doses were based only on larvae that were motile. Most of the larvae were straightened and obviously dead, whereas others were coiled but still considered to be non-infectious. For the remaining experimental infections the doses were based on motile and coiled larvae, and only straight larvae were considered dead and non-infectious.

DNA extraction

Genomic DNA from *H. contortus* has been extracted for a number of studies from a total of 486 individual adult worms. Different methods were used depending on the quality requirements of the DNA, previous storage of the worms (fresh, frozen or alcohol preserved) and time efficiency.

For most worms we used the QIAmp Tissue Kit (Qiagen). Individual adult worms were treated according to the manufacturer's protocol. Each DNA sample was eluted in 400 µl Buffer AE (10 mM Tris-HCl; 0.5 mM EDTA; pH 9.0) from which 1-3 µl was used as template in the PCR. This protocol was used for 30 worms studied in paper I. In addition, the DNA from all 150 individual worms in paper II was also prepared using this kit, but this time with an additional treatment of RNase A (1.7 mg/ml) to obtain RNA-free samples.

A DNA extraction robot was used to prepare the templates for the remaining 80 of the worms used in paper I. Briefly, individual worms were lysed in 100 µl lysis buffer (50 mM Tris pH 8, 100 mM NaCl, 100 mM EDTA, 1% SDS) and 25 µg Proteinase K overnight at 37 °C. The DNA was extracted with a GenoM-48 Robotic Workstation (GenoVision AS) using the GenoPrep-DNA from Tissue Kit (GenoVison AS) according to the manufacturer's protocol. Each sample was eluted in 300 µl ddH_2O, from which 1 µl was used as template in the PCR.

It is known to be difficult to extract PCRable DNA from long-term ethanol preserved nematodes. However, GeneReleaser (Bioventures) works, and has been used for samples stored in alcohol prior to DNA extraction. Single worms were put in 20 µl of PCR buffer (x1) and incubated at 70 °C for 10 minutes. Thereafter the worms were digested with 2 µl proteinase K (10 mg/ml) for at least three hours at 56 °C. A volume of 20 µl of GeneReleaser was added and the tube was microwaved for 6 minutes at 750 watts (4500 watt-minutes). The supernatant containing the purified DNA was collected and stored at 4 °C until use. A volume of 1 µl extracted DNA was used for PCR. This method was used on worms in paper IV to verify their origin (see 'verification of worm origin' in Materials and Methods).

Worm recovery

Stable trial (paper III)

At *post mortem*, the abomasum and abomasal mucosa of each animal were processed according to Donald *et al.* (1978) and Dobson, Waller & Donald (1990). The abomasum was cut open and the mucosa was carefully rinsed with tap water. The content from each individual was collected into separate buckets and filled up to 2 liters with water. The solution was stirred carefully, and four 20 ml samples were taken and stored at –20 °C until the samples were examined. Thus, each worm in those samples represented 100 worms in the abomasum.

To recover inhibited larvae embedded in the abomasal mucosa, the mucosa was scraped and collected in plastic containers. A digestion fluid composed of 10 g pepsin and 17 ml concentrated HCl per 1000 ml water was freshly prepared, and 250 ml of this digestion fluid was then added to each mucosa. The solution was incubated for approximately 6 hours at 37 °C with occasional mixing. The solution was mixed with water to a final volume of 2 liters, and four 20 ml samples were removed and stored at –20 °C until the samples were examined with a sensitivity of 100 worms (Dobson, Waller & Donald, 1990).

Both kinds of samples were examined from each animal using a dissecting microscope (Olympus SZX9). Worms were counted in 20 ml aliquots of the wash/digest with a minimum detection limit of 100 worms, and were differentiated with respect to sex and developmental stage.

Field studies (paper IV)

Two different methods were used for worm recovery in the tracer lamb studies.

Lövsta and Malmö

The method used in the overwinter survival study on artificially infected pastures in Lövsta and Malmö aimed to detect all worms that had reached the adult stage. The lambs were slaughtered, and at autopsy the total content of the abomasum from each lamb was collected and the abomasal mucosa was washed carefully. The total collection was passed through a series of sieves with the finest mesh of 250 μm. This mesh size is enough to retain all adult parasites. The retained material on all the sieves was preserved in 50% ethanol and refrigerated until examination. The material was examined as described above.

There were two drawbacks with this method: i) the tracer animals were taken straight off pasture, and so all worms that were picked up the last three weeks of the study were missed in worm recovery, as they had not had the chance to develop to adults. On the other hand, the tracer animals were allowed to graze for a period of 6 weeks, and any larvae picked up during the first three weeks and that developed into adults would have been recovered; ii) worms that stayed inhibited would not be detected with this method.

Bergshamra and Vomb

For the field studies in Bergshamra and Vomb the methods of Donald *et al.* (1978) and Dobson, Waller & Donald (1990) were used (for a detailed description see ‘stable trial’ above). With these methods all developmental stages of the parasite will be detected, although with a somewhat reduced sensitivity. In these grazing trials, the tracer lambs were allowed to graze for three weeks. The tracer lambs were also allowed a housing period of three weeks before slaughter, to prevent auto-infection and to allow all worms to develop through to adults. The drawback with this method, as compared to the worm recovery method used at Lövsta and Malmö, is that it has a detection limit of 100 worms, which clearly is a disadvantage when worm burdens are low.

Verification of worm origin

In paper IV we studied the overwinter survival of L3s on pastures contaminated the previous grazing season by artificially infected animals. The infective larvae used for this study were of either Kenyan or Swedish origin. The aims were both to investigate the overwinter survival for *H. contortus* in Sweden and to compare the results between the two isolates in the event of survival. A small fraction of overwinter survival was observed for both isolates (paper IV). To verify the origin of the worms recovered from the tracer lambs, and to ensure that no cross contamination had occurred between the adjacent pastures, worms that were recovered were genotyped. This was done using the marker in ITS-1, position 134, of the rDNA described in paper I. Up to 8 adult worms from each of the four pastures (Lövsta infected with Swedish larvae, Lövsta infected with Kenyan larvae, Malmö infected with Swedish larvae, Malmö infected with Kenyan larvae) were analyzed.

Briefly, the DNA was extracted from the worms and ITS-1 was amplified. The amplicons were cloned, and 5-8 positive clones were sequenced from each individual worm. All (100%) worms picked up on pastures contaminated by the Kenyan isolate lacked the insertion at position 134 in all sequenced clones, whereas 80% of the worms recovered from tracers kept on pastures contaminated by the Swedish isolate had an insertion at this position. This molecular verification could only be performed on very few worms, due to the lack of parasite material. Still, our main interest with this investigation was to rule out the possibility that the Swedish worms had contaminated the Kenyan pastures. The results strongly indicate, according to the results presented in paper I, that the worms picked up on the pastures originate from Sweden and Kenya, respectively, and that cross contamination between the pastures is unlikely.

Pyrosequencing assay, SNP identification and analysis

In paper I we investigate the species identity of *Haemonchus* from sheep and goats in Sweden as well as from Kenyan sheep through analysis of variation in sequence data of ITS-1, ITS-2 and the 5.8S rRNA gene. Variable sites (single nucleotide polymorphisms, SNPs) were first found between the two isolates through dideoxy sequencing. These putative fixed differences between the isolates were further analysed in a larger set of individual worms, to verify that these differences were consistent. The main advantage of SNPs is their high potential for high throughput analysis (Zhang & Hewitt, 2003).

We choose to use Pyrosequencing™ technology to verify these SNPs. Advantages with this approach are that the Pyrosequencing assay allows the estimation of SNP allele frequencies using peak height estimates (Berg, Sanders & Alderborn, 2002) while at the same time enabling high throughput of samples. This is important, as incomplete rRNA repeat homogenization, which may lead to intraindividual differences, has been observed in related trichostrongylids (Nadler *et al.*, 2000b).

Pyrosequencing is a real-time sequencing method designed for the identification of SNPs (Ahmadian *et al.*, 2000; Fakhrai-Rad, Pourmand & Ronaghi, 2002). The technology is based on the detection of light released as a consequence of a cascade of enzymatic reactions following the incorporation of a nucleotide (Fig. 4) (Ronaghi *et al.*, 1996; Ronaghi, Uhlen & Nyren, 1998):

- In this sequencing-by-synthesis method all four nucleotides are added stepwise to the template hybridized with a primer. If the nucleotide is complementary to the base in the template strand, it gets incorporated into the DNA strand by DNA polymerase. The incorporation is followed by release of pyrophosphate (PPi) equal in molarity to the amount of incorporated nucleotide.

- The released PPi is converted to ATP by ATP sulfurylase, and the concentration of ATP is then sensed by luciferase.

- The light produced in the luciferase-catalyzed reaction is estimated by a charge coupled device (CCD) camera and detected as a peak in a pyrogram. Each light signal is proportional to the number of nucleotides incorporated.

- Unincorporated nucleotides and the produced ATP are degraded between each cycle by apyrase, a nucleotide degrading enzyme.

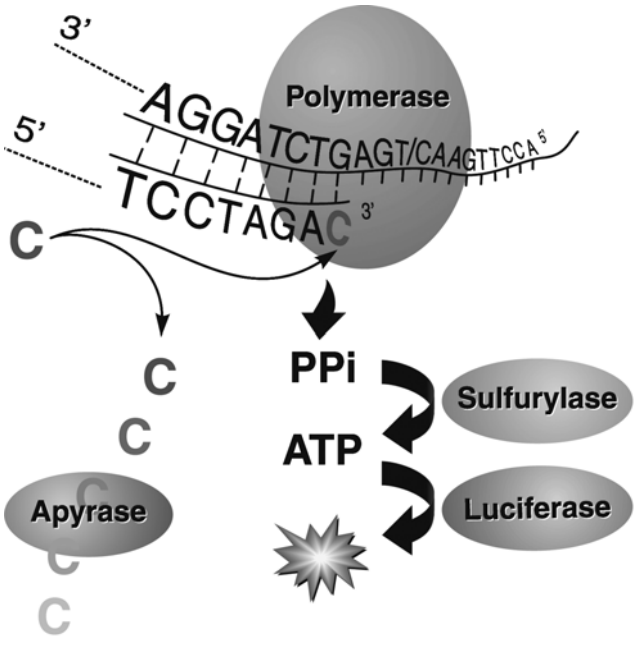

Fig. 4. Principle of Pyrosequencing.

Amplified Fragment Length Polymorphism, AFLP

In paper II we examined the population genetic structure (PGS) of *H. contortus* by assessing the genetic variability using both amplified fragment length polymorphism (AFLP) and *nad4* sequences of the mitochondrial genome. Details on advantages and disadvantages for these markers are given in the Background.

The AFLP technique is based on the amplification of subsets of genomic restriction fragments using PCR (Vos *et al.*, 1995). The AFLP procedure involves several steps (Fig. 5) (Krauss & Peakall, 1998):

- Digestion of DNA with restriction enzymes and ligation of adaptors. The digestion of the DNA is performed with two restriction enzymes, preferably a hexa-cutter (rare) and a tetra-cutter (frequent). In paper II we used *Eco*RI and *Mse*I. The double-stranded adapters are ligated to the ends of the DNA-fragments, to generate template DNA for amplification. The sequence of the adapters and the adjacent restriction site serve as primer binding sites for subsequent amplification of the restriction fragments.

- Preselective amplification. PCR amplification of restriction fragments is achieved by using the adapter and restriction site sequence as target sites for primer annealing.

- Selective amplification. Selective nucleotides are included at the 3' ends of the PCR primers, which therefore can only prime DNA synthesis from a subset of the restriction sites (for details on the primers used in paper II see Materials and Methods in the manuscript). Only restriction fragments in which the nucleotides flanking the restriction site match the selective nucleotides will be amplified.

- Analysis of the amplified fragments by electrophoresis on a capillary system.

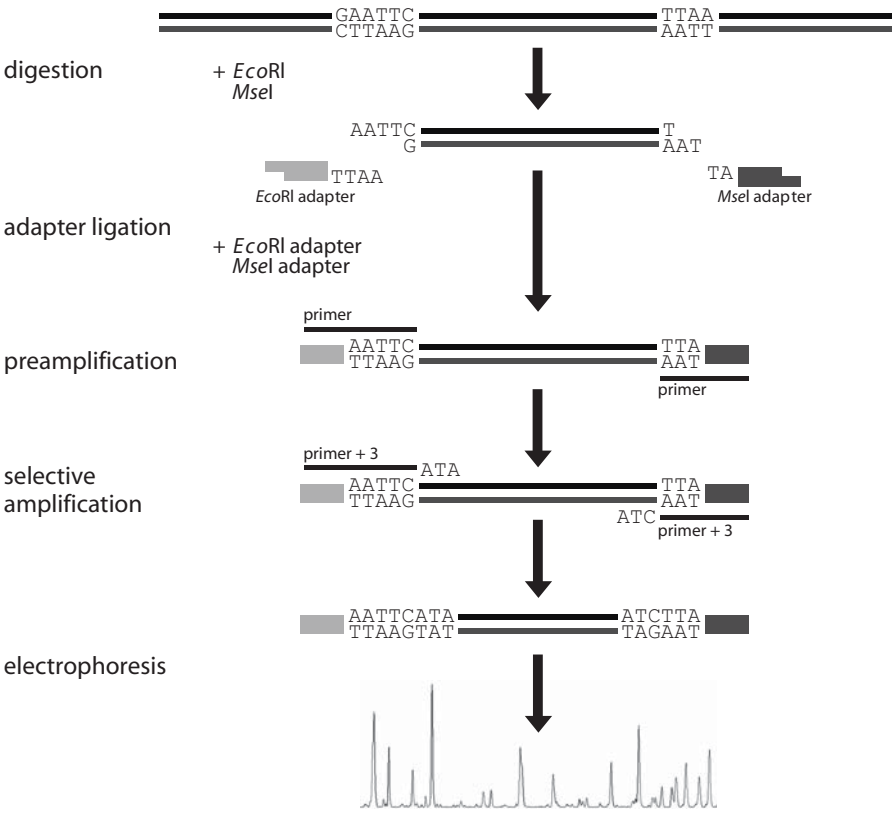

Fig. 5. The principles of amplified fragment length polymorphism (AFLP).

Phylogenetic analyses

Phylogenies, or phylogenetic relationships, are in general patterns of shared common evolutionary history between biological replicators, such as species or genes, and are given as sister-group relationships (Felsenstein, 2003). Molecular phylogenetics is the study of evolutionary relationships among taxa or genes, and is a combination of molecular and statistical techniques (Felsenstein, 2003). In general, organisms with more similar genes are more closely related, *i.e.* they are likely to share a more recent common ancestor than are distantly related taxa (Salemi & Vandamme, 2003).

The relationships between species can be represented by a phylogenetic tree, which is a graphical representation with nodes and branches in a bi- or multifurcating structure. Methods to construct phylogenetic relationships can be classified into two groups, *i.e.* cladistic methods (maximum parsimony, maximum likelihood and bayesian) and phenetic methods (distance matrix methods such as neighbor joining).

In paper II we used maximum parsimony, neighbor joining and minimum evolution to infer the phylogenetic relationships between 19 isolates of *H. contortus*. The distances for the two latter methods were calculated differently depending on if the marker was dominant or codominant (for details see Materials and Methods in paper II). In the paper we choose to present the minimum evolution tree because the simplest pattern was presented in this tree, and for comparison describe the differences to the other trees calculated (Table 3, paper II).

However, phylogeny *sensu stricto* studies the hierarchical relationships between genes from different species (Posada & Crandall, 2001). By contrast, relationships between genes sampled from individuals within a species are tokogenetic *i.e.* not necessarily hierarchical as they result from sexual reproduction (Posada & Crandall, 2001). Over the last 15 years there has been increased development of methods to construct networks (*i.e.* trees that allow reticulations among the branches rather than strictly bifurcating structures) (Morrison, 2005). It has been argued that the more traditional methods originally developed to estimate interspecific relationships may violate some of their assumptions when applied on intraspecific data. That could lead to poor resolution, or may portray inadequate genealogical relationships (Posada & Crandall, 2001). Even though use of networks would overcome some of these disadvantages, other problems can arise. Currently there is no method to create networks that display an evolutionary diagram (Huson & Bryant, 2006). However, network approaches can incorporate population processes and account for conflicts in a dataset. A network does not attempt to force data onto a unique tree, but rather provides an indication on how tree-like the data are. Only for ideal data will this be drawn as a tree. When the data contains conflicting signals, the data will be drawn as cycles or reticulations in the graph (Huson, 1998). The conflicts can be due to one of three reasons: 1) model misspecification 2) analogy 3) homology, the latter showing the "true" evolutionary history. The major problem is to work out which reticulations are caused by homology rather than misspecification or analogy. For this reason, phylogenetic networks are yet not commonly used.

In addition to the phylogenetic trees calculated in paper II, we therefore constructed a network of the relationships among the 150 individuals sampled in the AFLP dataset (Fig. 6). The network was estimated using the same restriction distances calculated for the phylogenetic trees, and calculated as a neighbor-network using the SplitsTree program (Huson & Bryant, 2006).

The network shown in Fig. 6 clearly shows the same basic relationships as presented in paper II (Fig. 1 and Table 3, paper II). However, the conflicts between the trees discussed in paper II can also be observed in the network. For example, the two worms Grek275 and Ken192 that do not cluster with the rest of their isolate in the neighbor-joining and maximum parsimony analyses can also be observed as distinct branches between groups in the network. The rest of the individuals from the same isolates were clustered both in the phylogenetic analyses and in the network.

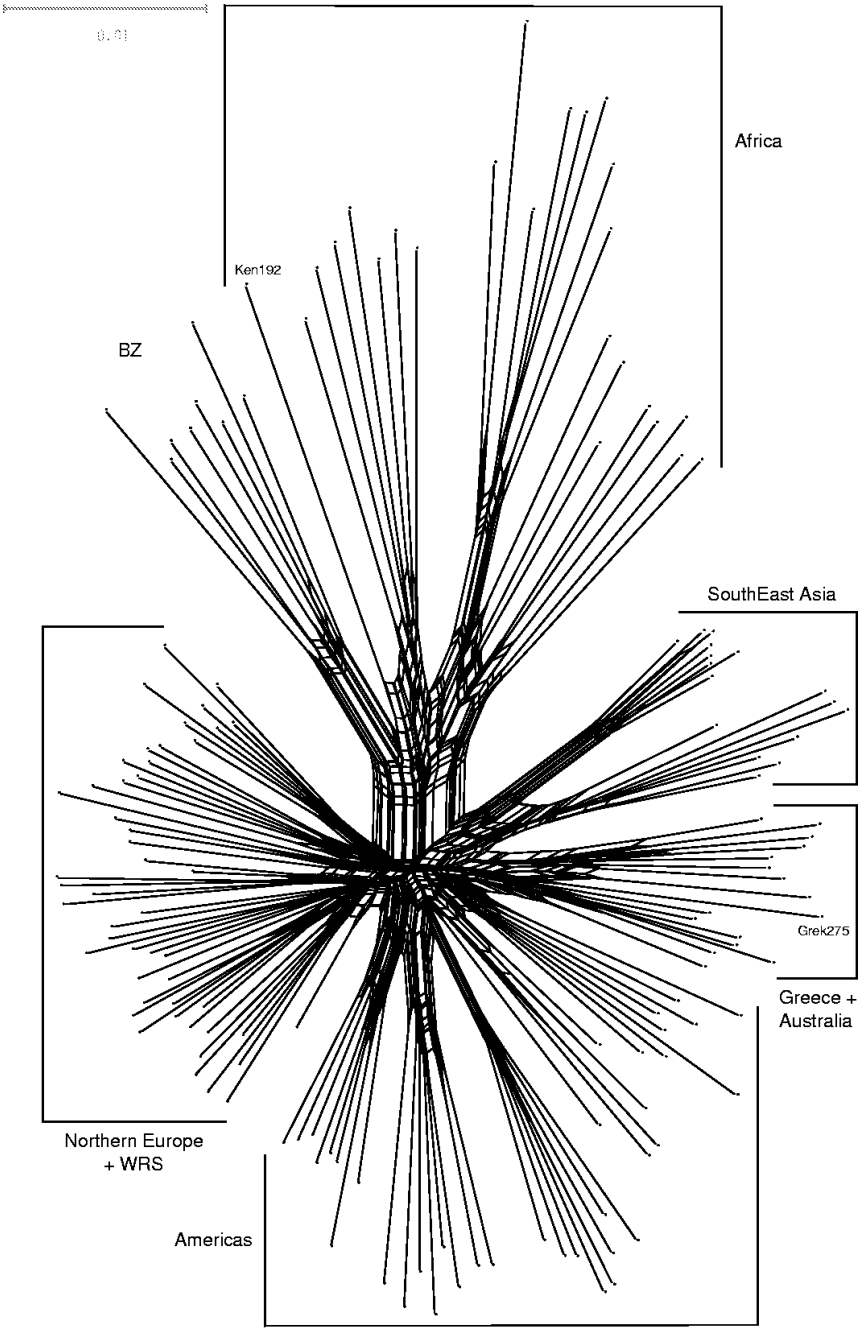

Fig. 6. Inferred neighbour-net network of amplified fragment length polymorphism (AFLP) data for *Haemonchus contortus* from 19 isolates representing all continents. Conflicts among the characters are shown as reticulations in the diagram. The scale is the inferred number of nucleotide substitutions.

Results and discussion

Understanding the causes and mechanisms of local adaptation, and how it shapes genetic diversity, has been one of the major challenges in evolutionary biology. Spatial environmental heterogeneity is repeatedly established as a factor contributing to creation of genetic heterogeneity, and fluctuations in biotic and abiotic factors over time have been found to have large impact on populations and species (Hedrick, 1976; Reznick & Ghalambor, 2001). Thus, climatic factors are potential sources of selection on different phenotypic traits.

Even though *H. contortus* is an internal parasite, the completion of its life cycle is dependent on the external environmental conditions, as development and survival of the egg and first three larval stages are influenced by temperature and humidity. On this basis, the environmental conditions in which external larval stages of the parasite can develop and complete its life cycle are limited in Sweden. Accordingly, we investigated whether *Haemonchus* found in Sweden is the same species as the one found in the tropics, and what adaptations this species may have acquired to survive this cold temperate climate.

Species identification (I)

All species concepts aim to bring order to life. Even though a species definition may or may not make reference to characters (these characters may or may not mean morphological characters) all concepts use character data to adjudicate species boundaries.

Molecular prospecting with comparison between intraspecific and interspecific differences has been widely used when defined species have been studied, and also when cryptic species have been suspected. A first prospecting for cryptic species might be to look for unusually large genetic distances in different molecular markers (Vilas, Criscione & Blouin, 2005), usually called the genetic yardstick approach (Nadler, 2002). However, the genetic yardstick approach has been questioned for species delimitation in favour of the search for fixed differences (Nadler *et al.*, 2000a; Nolan & Cribb, 2005).

While investigating the species status of *H. contortus* from Sweden, we decided to ignore the genetic yardstick approach and include only fixed differences between the different isolates we studied. Accordingly, we conclude that *Haemonchus* isolated from Swedish sheep and goats belong to the same species, *H. contortus*, as does the studied isolate from Kenya (paper I). In our work, small variations were observed in the ITS-1 and ITS-2 sequences of the worms sequenced. The magnitude of this intraspecific sequence variation was similar for both regions and ranged 0.9-1.8% in ITS-2 and 0.8-1.8% in ITS-1. These results are similar to earlier results for the ITS-2 in *H. contortus* of livestock ruminants. For example, Stevenson, Chilton & Gasser (1995) found no intraspecific variation in *H. contortus* of sheep and *H. placei* of cattle. In contrast, Heise, Epe &

Schnieder (1999) recorded an intraspecific variation of 2.6%, corresponding to 6 nucleotide positions, in a study where the ITS-2 sequence of *H. contortus* was cloned and sequenced.

In our case, two positions (134 and 402 in the ITS-1) appeared to differentiate between the worms isolated from Swedish sheep and goats compared to Kenyan sheep. However, no fixed characters were identified when Pyrosequencing technology was applied on the putative fixed differences first identified with dideoxy sequencing. The two positions analyzed with Pyrosequencing technology both represent positions with intraindividual differences, *i.e.* heterogenetic sites that differ between the different copies in the multigene family. This incomplete rRNA repeat homogenization has been observed in other related trichostrongylid nematodes (Hoste *et al.*, 1995; Nadler *et al.*, 2000b).

Population status (I and II)

The isolates from Sweden and Kenya were genetically distinct populations (papers I and II). Results from the Pyrosequencing analysis showed four distinct genotypes from which the geographical identity could be distinguished with only minor exceptions (paper I). The insertion at position 134 in ITS-1 was nearly exclusive for the Swedish isolates (irrespective of sheep or goat host origin). Of the 40 worms from Kenya, only one (2.5%) worm had a peak in the pyrogram for this SNP. The majority (82.5%) of the Kenyan worms could be identified with a C/A heterogeneity at position 402 in the ITS-1. This marker, combined with the lack of insertion at position 134, was exclusive for the Kenyan isolate (Table 2, paper I).

AFLP analysis and *nad4* sequencing further developed our understanding of the population structure of these isolates (paper II). We found, in contrast to previous population genetic studies of *H. contortus* (Blouin *et al.*, 1995; Blouin *et al.*, 1998), that both the variation in the AFLP data and in the mtDNA marker revealed high levels of differentiation between isolates and were characterized by strong population structure (Fst=0.18 and Nst=0.57). The AFLP showed the greatest discriminatory power between the two markers and clearly separated the individuals from different isolates into distinct clusters, both within countries and between continents. This is most probably due to the better representation of the genome by the AFLP technique, since both data sets included exactly the same individuals, and the differences in genetic structure are unlikely to reflect different population processes. The sequence variation in *nad4* showed strong population structure, but lacked a distinct geographical pattern in the phylogenetic analysis, *i.e.* this analysis does not provide enough information to allow inference of the regional origin of *H. contortus*. Accordingly, the variation in this mitochondrial gene was also too low to differentiate between populations within each continent.

A high degree of variability was observed in the AFLP dataset, which is in agreement with earlier studies, not only for *H. contortus* (Otsen *et al.*, 2001), but also for other parasitic nematodes of domesticated hosts (Höglund *et al.*, 2004;

Nejsum *et al.*, 2005). The high degree of diversity that we observed in *nad4* is in agreement with earlier published data on trichostrongylid nematodes (Blouin *et al.*, 1995; Braisher *et al.*, 2004; Fisher & Viney, 1998). However, there are clearly limitations to the population patterns that this locus can detect, and given the comparative ease of gaining large numbers of informative AFLP markers, the application of AFLP may be of more practical utility.

The overall genetic diversity does not seem to have decreased following laboratory maintenance in our laboratory isolates from Sweden and Kenya. In the phylogenetic work with 19 populations, these isolates cluster with the other isolates from their respective continents (Fig. 1 and 2, paper II). This indicates that the overall genetic variation is still representative of natural populations, and that it has not been dramatically changed following laboratory maintenance. This is in accordance with another study on *H. contortus*, where AFLP was used to show that the level of polymorphism across the genome was not significantly decreased following selection for drug resistance (Otsen *et al.*, 2001). This has also been shown in a study on the bovine lungworm, in which a laboratory strain showed more within-population variation than did most of the field populations under study (Höglund *et al.*, 2004). We therefore consider these isolates representative of the diversity encountered in natural populations and for the two contrasting climatic origins they represent in this thesis.

Taken together, we find the resolving power of the whole-genome fragment analysis to be greater than the mtDNA marker, even though mtDNA has proven particularly effective for detecting population structure in parasitic nematodes. The general conclusion from these two studies is that the two isolates studied throughout the work of this thesis represents two distinct ecological populations of *H. contortus*.

Evolutionary adaptation or phenotypic plasticity (III and IV)

Given that the isolates studied herein represent one species, we further hypothesized that the genetic variation may reflect an adaptation to the environmental heterogeneity that this species meets over its complete distributional range. Across the broad geographic scale that these two isolates represent, we should be able to observe different phenotypes if the species has adapted to the climatic variation. The term “genotypic adaptation” entails the evolution of specific mechanisms that improve the performance of an organism in a particular environment (Johnston & Bennett, 1996). This was one of the major points of Charles Darwin's theory of evolution by natural selection: organisms adapt, as a result of an evolutionary process, to their environment (Darwin, 1859). However, the prediction that populations differ in specific phenotypic traits shaped by the climatic differences from their geographical origin could not be demonstrated (papers III and IV).

These studies included analysis of phenotypic traits of both the pre-parasitic stages as well as of the adult parasites. No differences in development and survival

were found between the two isolates of *H. contortus* in the pre-parasitic stages (paper IV). The development from egg to larvae was studied at several different temperatures under preset laboratory conditions. As has previously been shown (Crofton, 1965; Rossanigo & Gruner, 1995; Silverman & Campbell, 1959), the development was most successful at the highest temperature (15 °C with following incubation at 25 °C) and no significant differences in development between the two isolates were observed at any of the studied temperatures.

In contrast, low temperature (5 ºC) favoured the long-term survival of infective larvae stored for up to 6 months in culture flasks in climatic chambers (paper IV). However, no significant differences in this respect were observed between the Swedish and Kenyan isolates. The fact that larvae died quickly towards the end of the experiment may be due to the fact that nematode infective larvae utilize lipids as their primary energy source (Nwosu, 1979; Wilson, 1976), and the dramatic change in survival rate after 16 weeks is likely to be due to the exhaustion of food reserves rather than intolerance of temperature.

Adaptation to different climatic origins of the two isolates could not be shown in a range of phenotypic traits at the infection level used (paper III). These conclusions were drawn from comparisons of establishment, male:female ratio, inhibition pattern, prepatent period, and worm length between the two isolates of *H. contortus*.

However, significant differences were observed between these isolates, both with respect to prepatent period and larval inhibition when fresh larvae were used for infection (paper III). The prepatent period differed by two days between the Kenyan and Swedish isolate (17 and 19 days respectively). However, the literature on *H. contortus* in sheep reports prepatent periods that differs greatly (12-21 days) (Thamsborg, Søland & Vigh-Larsen, 2001; Urquhart *et al.*, 1996). In addition, there is an inherent shortcoming in the faecal egg count procedures used. Also, the diurnal fluctuations in egg output by the parasite and the daily variations in rate of digestion are factors that can explain this apparent difference in prepatent period. However, in our attempt to cover for these shortcomings, four samples were independently taken and analyzed from each animal daily.

Both isolates underwent inhibition, whereas only *H. contortus* of the Kenyan isolate was significantly influenced by the storage conditions of the larvae (paper III). The observed levels of inhibition were somewhat surprising, not least in the Kenyan isolate. However, high levels of inhibition in young lambs artifically infected with *H. contortus* has been reported earlier (Connan, 1975; Hrabok *et al.*, 2006; Ogunsusi & Eysker, 1979). It has been suggested that seasonal change could be the causative factor of inhibition in *H. contortus* (Connan, 1971; Muller, 1968). In contrast, Waller & Thomas (1975) suggested that inhibition in *H. contortus* is an obligatory genetic strategy for survival rather than dependent on external stimuli. The lack of correlation between storage and inhibition rate in lambs given larvae of the Swedish isolate supports the view that the mechanisms behind inhibition are not triggered by environmental stimuli such as cold temperatures.

The results presented in this thesis suggest the occurrence of phenotypic plasticity in *H. contortus* as a response to different environments and storage conditions of infective larvae prior to infection (papers III and IV). The term phenotypic plasticity is used to describe the ability of a genotype to express different phenotypes in response to different environments, *i.e.* an acclimation to new conditions for an organism within one generation (Bennett & Lenski, 1997; Johnston & Bennett, 1996; Little, Watt & Ebert, 2006).

Significant differences were found depending on the storage conditions of infective larvae, especially with respect to prepatent period and establishment (paper III). Although the prepatent period was significantly longer after inoculation with larvae that had been cold treated prior to inoculation (20 days compared to 18 days for fresh larvae) these differences could be accounted for by several other factors as discussed above.

The observed worm establishment in the experimental infections performed in paper III was high. It ranged from 43-74% depending on storage conditions of the infective larvae prior to inoculation, where the higher establishment was for the cold-treated L3s. This could be interpreted as if *H. contortus* exhibits a superior fitness if infective larvae are subjected to cold stress prior to infection. However, this is highly unlikely. A more probable explanation is that this was due to a miscalculation when we inoculated the lambs. At worm recovery, more than 2000 worms (*i.e.* more than the calculated initial dose) were counted in some of the sheep infected with cold-treated L3s. This could to some extent be explained by the fact that the methods used, both to estimate the number of larvae for the inoculations and to enumerate established worms, are rough estimates. Since the larvae used for experimental inoculations were considered as infective based on larval motility (see details in 'larval doses' in Materials and Methods), the infection doses could easily have been underestimated. This may result in differences in accuracy of calculation between the two experiments, as 100% of the larvae were motile at the time of inoculation in the fresh samples. In contrast, in the experiment with cold-treated L3s up to 35% of the larvae were non-motile and therefore regarded as non-infective. Consequently, for the experiment when larvae had been cold-treated prior to infection each lamb may have received a slightly higher dose of larvae than calculated.

In addition, great variation in establishment rate has earlier been reported in the literature for *H. contortus* (Waller & Prichard, 1986). Waller & Prichard (1986) summarize the huge differences in establishment rate for specific parasite isolates (McMaster and VSRG *H. contortus*) in artificial infections given to young lambs. In conclusion, the inability to ascertain the infectivity of the larvae, in addition to the fact of difficulties to make up larval doses and dosing procedures, could entirely account for the apparent superior infectivity of the cold-treated larvae.

The role of genetic variation and phenotypic plasticity in adaptation of *H. contortus* to different climatic origins was also studied for another phenotypic trait, the overwinter survival of L3 on pastures. No major differences in the survival capacity of the free-living infective larvae on pasture were noticed

between the isolates of Swedish or Kenyan origin when studied over two grazing seasons (paper IV). The overall survival of L3 on pasture was low, and no significant differences were observed between the isolates on either geographical location (southern Sweden and central Sweden). However, the results presented in paper IV provide 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 overwinter survival. These experiments confirm the current opinion that *H. contortus* does not survive well as L3 on pasture under Swedish winter conditions. However, low infections were recovered, indicating phenotypic plasticity.

Survival strategies (IV)

This tropical parasite appears to exhibit phenotypic plasticity rather than having undergone evolutionary adaptation to survive this cold temperate climate (paper IV). Although the development from egg to larvae was low, both at a constant temperature of 5 °C and when the temperature was cycled from –1 to 15 °C over one day, development does occur at these low temperatures for both isolates during the first few weeks. This is in contrast to earlier results by Silverman & Campbell (1959), who showed that little or no development of eggs take place below 9 °C. On the other hand, the results after week 2 indicated that the eggs died, as no, or very low, development occurred when faeces were incubated at 5 °C and –1 to 15 °C, and thereafter incubated at 25 °C for an additional week. This is in agreement with previous studies, where unembryonated eggs of *H. contortus* survived at temperatures below 1.1 °C for only 3-7 days (Silverman & Campbell, 1959). Although the results presented here indicate a stronger resistance to cold than reported earlier, the eggs were, irrespective of origin, very sensitive to cold stress, and we were also unable to demonstrate that they survived on pastures long enough to significantly contribute to transmission between grazing seasons. On the other hand, the long-term survival under stressful conditions (–1 to 15 °C) indicates that the infective stage of *H. contortus* be slightly more resistant to cold temperatures (paper IV) than previously reported.

In addition, in paper IV we demonstrated that this parasite survived, although in low numbers, over winter on pastures in Sweden. Several field trials have been performed. In these, *H. contortus* was picked up on pasture at three of four locations (Malmö, Lövsta and Vomb) following winters over two different seasons (2001-2002 and 2002-2003). However, in Vomb we found *H. contortus* in only one of the tracer lambs. On the other hand, in this study *Teladorsagia circumcincta* was ubiquitous (approximately 115 000-200 000 worms), and low densities of *H. contortus* could therefore have been overlooked. Despite this, we found 1250 inhibited larvae of *H. contortus* in one host animal.

Overwintered larvae were also picked up where experimentally infected sheep had contaminated the pastures during the previous season. In this experiment (Malmö and Lövsta), the degree of contamination of the pastures during 2001 was

influenced by the low stocking-rate (2 ewes and 2 lambs on 0.8 ha). In addition, at the time of slaughter localized areas of the pasture plots in Malmö were covered by knee-high grass, which lead to a dilution effect of the infective larvae in the herbage. Still, *H. contortus* of both isolates obviously survived the winter, as larvae on pasture as adult worms were found in tracers from all plots on both study sites. As earlier discussed, the method used for the recovery of worms was selected to enumerate the total adult *H. contortus* burden in sheep after a defined grazing period of 6 weeks (see 'worm recovery' in Materials and Methods). The idea was to increase the sensitivity of the method, and to detect any larvae picked up during the first 3-4 weeks that had developed into adults. However, the results from the Vomb study (for details see Waller *et al*., 2004a) showed that 100% of the *H. contortus* recovered in May 2003 were in the fourth larval stage. Thus, it cannot be excluded that the tracer lambs in the Malmö/Lövsta study also had a greater part of the infection inhibited. These internal larval stages were obviously missed with the worm recovery method used. Consequently, the actual densities of established worm burdens may have been underestimated.

Taken together, we conclude that contaminated pastures can support low numbers of *H. contortus* larvae at turnout the following year. However, it was also demonstrated that the majority of worms picked up were in the fourth inhibited larval stage. Inhibited populations of *H. contortus* at this time of the year are of no immediate importance in perpetuating the parasite for the forthcoming season. These results indicate that overwintering of larvae on pasture in Sweden was of limited significance. Accordingly, overwintering on pasture is not the most important method for seasonal transmission of *H. contortus* in Sweden.

The low overwintering survival may be of limited significance for *H. contortus* epidemiology, but it should not be ignored completely. One must bear in mind that *H. contortus* has a very high fecundity, with female worms producing up to 10 000 eggs per day (Coadwell & Ward, 1982; Coyne, Smith & Johnstone, 1991; Thamsborg, Søland & Vigh-Larsen, 2001). Accordingly, such high egg production this may lead to a quick build up of the larval population on pasture. In both Malmö and Lövsta gravid adult females were found in several animals. One can only speculate as to what extent this would lead to problems for the grazing animals later in the season. However, it can be concluded that, as long as *H. contortus* does survive winters on pastures in Sweden and develop through to adults, this will complicate attempts to eradicate this parasite from farms.

Concluding remarks

This thesis aimed to add knowledge about *Haemonchus* in Sweden. This well-studied parasite has long been thought of as a tropical and subtropical problem, although sheep farmers in temperate regions have considered it a threat to their animals for several decades. The parasite is thus an emerging problem in Sweden. Knowledge about how this parasite responds to local climatic factors can help in developing advice on control strategies, which in turn will lead to better animal health.

The results gathered within the frame of this thesis show that *Haemonchus* in small ruminants in Sweden represents the same species as in the rest of the world, *Haemonchus contortus*. The genetic variability between *H. contortus* in different hosts in Sweden showed that sheep and goats share the same species of *Haemonchus*, whereas cattle have their own species *H. placei*. This is the first time *H. placei* has been described in Sweden. The fact that cattle have their own species of *Haemonchus* may oppose the former conclusion that cattle are of importance to transmission of this parasite to sheep. However, this is based on a single finding, and further attention to this issue is required.

H. contortus in Sweden does not seem to have undergone any major genotypic adaptation to survive this unfavourable environment, based on studies on several phenotypic traits such as infectivity, prepatent period, inhibition and overwinter survival. The results presented in this thesis indicate that this species has a large phenotypic plasticity, and that it can avoid environmental stress by exhibiting different epidemiological strategies depending on the surrounding conditions for the pre-parasitic stages.

In contrast to what has earlier been thought, it was evident that *H. contortus* can survive winters as L3 in small numbers on pastures in Sweden. However, there are many factors influencing the occurrence and significance of this overwinter survival. Based on the results provided in this thesis, we cannot provide sheep holders with the final answer as to when to consider pastures to be worm-free. However, it can be concluded that the main transmission between grazing seasons was as inhibited larvae within the pregnant ewe.

A common prediction has been that host movements may be a major determinant for parasites with limited dispersal capacity in their free-living stages. However, our values for the population structure indicate that we have detected more genetic structure than has previously been reported for most nematodes of domesticated hosts. This suggests that many of the assumptions about the nature of population structures in parasites of such hosts may be overly simplistic. We suggest that one explanation to the strong population structure observed between the different Swedish isolates could be due to the poor overwinter survival of *H. contortus* L3 on pasture in Sweden, resulting in extreme bottlenecks for this parasite's external life stages.

Future Research

Advice on strategic parasite control of *H. contortus* in Sweden is today based on the assumption that this parasite does not survive on pastures over winter, and that one cold season is enough to give *Haemonchus*-free pastures to grazing animals. Even though this thesis shows some capacity for overwinter survival of *H. contortus* in Sweden, it does not provide the final answer as to what extent this is of any practical importance. Given the results presented herein, together with this parasite's very high fecundity, I suggest that it would be worthwhile to further investigate the survival capacity and its importance to *Haemonchus* epidemiology in Sweden.

The work presented here indicates that *H. contortus* can tolerate environmental stress and complete its life cycle due to phenotypic plasticity, rather than to genotypic adaptation in specific geographical regions. Cold stress seems to have less effect on the infective larvae than was first thought. It would be of interest to further investigate at a molecular level the response of L3s to cold stress. This could, for example, be done in a study of differentially expressed genes in fresh larvae and long-term cold-treated larvae.

The spread of *H. contortus* in Sweden took new dimensions after the local development of anthelmintic resistance at the beginning of the 1990`s. Today, chemical control of *H. contortus* is dependent on anthelmintics other than the benzimidazoles, as resistance is a widespread problem throughout the whole country. Therefore, I find it highly relevant to study the resistance status to other substances, and in particular towards the macrocyclic lactones both at clinical and molecular levels.

Furthermore, the population genetics of Swedish *H. contortus* has been studied within the frame of this thesis. In paper II we included four isolates from Sweden. A broader knowledge of the PGS of this parasite is of great relevance for understanding the transmission biology between farms and the potential for spread of anthelmintic resistance. In addition, because the long-term survival of this parasite on pasture is limited, it would be of interest to study the differences between this and a cold-tolerant parasite's PGS, to further investigate whether the climate has an influence on the population structure, as suggested.

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