Do sheep worms occur in wild hares and rabbits in Australia?

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Declaration

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Abstract

Areas of common grazing between hares (*Lepus europaeus*), rabbits (*Oryctolagus cuniculus*) and sheep (*Ovis aries*) are widespread in south eastern Australia. For much of the year, lagomorphs are exposed to the infective larvae of the nematode parasites of livestock on farm pastures. Given that gastrointestinal parasites are a major problem for sheep graziers and that in experimental circumstances sheep helminths are able to develop in rabbits and hares, free-living lagomorphs were investigated regarding carriage of ovine nematode parasites under field conditions. 110 hares and 88 rabbits were shot by hunters in paddocks previously grazed by sheep or in vineyards near sheep pastures. Lagomorphs were acquired from November 2010 to August 2012 from the Adelaide region of South Australia, the western district of Victoria and central western New South Wales. Total helminth counts and examinations of spicule morphology were performed. PCR was utilized to confirm findings. My study revealed that the ruminant worm, *Trichostrongylus colubriformis*, is common in hares (prevalence 32.7%) and also, occasionally, occurs in rabbits (prevalence 3.4%). Statistical analysis showed no significant effects of age or sex of either hares or rabbits, in prevalence of worms ($P > 0.05$). Chi-Square and Fisher Exact tests were performed and showed that, in general, nematode parasite infestations were not significantly different in hares or rabbits ($P > 0.05$) for all regions examined. However, while the ruminant nematode *T. colubriformis* occurred more frequently in hares, rabbits were more commonly infected with the lagomorph-specific *Trichostrongylus retortaeformis* (prevalence 61.4 %). The lagomorph worm *Graphidium strigosum* was mainly found in rabbit stomachs obtained from New South Wales. The ruminant nematode *Trichostrongylus rugatus*, was identified infecting four hares and one rabbit from the Adelaide region, South Australia, and is reported for the first time in wild lagomorphs. Cross-transmission of nematodes between lagomorphs and sheep in the natural environment is much more prevalent than previously believed. Further studies will contribute important information to assist sheep producers manage nematode gastrointestinal parasites and may also lead to newly identified causes for the declines of lagomorph populations in various parts of the world.

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Chapter 1

Literature Review

1.1 Wild Lagomorphs in Australia

Two wild lagomorph species occur in Australia, the European rabbit (*Oryctolagus cuniculus* Linnaeus, 1758) and the European brown hare (*Lepus europaeus* Pallas, 1778) (Class – Mammalia, Order – Lagomorpha, Family – Leporidae, Fischer, 1817). Both species were introduced ca. 1860 by British settlers (Rolls, 1984). The rabbit represents a major pest in the continent and causes serious environmental and agricultural damage (Myers, 1986; Williams et al., 1995; Jarman and Stott, 2008). On the other hand, the hare, which is currently not at plague densities, is rarely subject to control (Stott, 2003a; Page et al., 2008). These wild herbivores have co-existed with livestock in pastures and open shrublands for many years. However, some important interactions between them have not yet been investigated.

1.2 Lagomorph parasitic nematodes

The helminth nematode fauna that naturally occurs in lagomorphs is diverse. The nematodes *Graphidium strigosum* (Allgoewer, 1997)**,** *Trichostrongylus retortaeformis* (Dunsmore, 1966; Boag, 1987a; Tenhu, 1998), and *Passalurus ambiguus* (Boag and Iason, 1986; Bordes et al., 2007; Beck and Pantchev, 2009) have been the most frequently recorded nematode species in free-living hares and rabbits, worldwide and in Australia. However, presumably because of a founder effect, a number of nematode parasites that occur in European hares and/or rabbits elsewhere on Earth do not occur in Australia, such as *Obeliscoides cuniculi*, *Trichuris leporis*, *Strongyloides* sp., *Protostrongylus* spp., *Nematodiroides zembrae* and *Dirofilaria scapiceps* (Evans, 1939; Bull, 1953; Mykytowycz, 1956; Hesterman and Kogon, 1963; Soveri and Valtonen, 1983; Boag, 1985, 1987a; Murray et al., 1997; Tenhu, 1998; Allan et al., 1999; Foronda et al., 2003; Newey et al., 2005; Bordes et al., 2007; Stojanov et al., 2008; Dubinsky et al., 2010; Tizzani et al., 2011; Lukešová et al., 2012).

1.3 Ovine parasitic nematodes

The sheep and wool industry contribute significantly to the Australian economy but ruminant gastro-intestinal nematode parasites (also known as "round worms") cause substantial economic losses estimated at $369 million per annum, making them the major sheep health concern (Holmes et al., 2006). The most important helminths which occur in sheep belong to the super-family Trichostrongyloidea (Lichtenfels et al., 1997) which occur in the gastro-intestinal tract (Durette-Desset, 1992; Chilton et al., 2006). The following are some of the most commonly found species of nematodes in sheep and represent a constraint to small ruminant producers worldwide: *Haemonchus contortus*, also known as the barber's pole worm; *Teladorsagia (Ostertagia) circumcincta* or brown stomach worm; and *Trichostrongylus* spp. or black scour worms. The first two species and *Trichostrongylus axei* are found in the abomasum (true stomach) of the host, other *Trichostrongylus* spp. occur in the small intestine (Cole, 1980) where *Nematodirus spathiger*, *N. filicolis*, *N. abnormalis* and *N. helvetianus* - thin-necked intestinal worms - are found (Beveridge and Ford, 1982), and whipworms such as *Trichuris ovis, T. globulosa* and *T. skrjabini* together with *Chabertia ovina* and *Oesophagostomum venulosum* are found in the large intestine (Pullman et al., 1988; Hutchinson, 2009). These major livestock parasites not only decrease weight gain and reduce quality of wool in sheep, but also cause significant animal mortalities and incur significant costs with treatment (McLeod, 1995).

1.4 Pathogenicity

Ovine trichostrongylosis is associated with a number of symptoms such as inappetence - food intake may be reduced by 20% (Holmes, 1985), anaemia and, very importantly, diarrhoea. Diarrhoea is a major problem for sheep producers because, besides the weight loss, the animals' wool gets soiled around the breech and this decreases its value and also makes them susceptible to myiasis (or "blowfly strikes") which can produce fatalities if left untreated (Heath and Bishop, 1986; Broughan and Wall, 2007; Jacobson et al., 2009; Williams and Palmer, 2012). According to Williams and Palmer (2012), sheep do not necessarily need to be heavily infested with worms to present diarrhoea. This can also occur as a result of the immune-pathological processes of the sheep itself, especially in animals of post-weaning age, which are mainly affected by the gastro-intestinal parasites *Teladorsagia circumcincta* and other members of Trichostrongylina. Large worm burdens of *Trichostrongylus* spp. can cause critical enteritis, severe villus atrophy, impaired villus/crypt ratios and hyperplasia of goblet cells (Pullman et al., 1991) as well as thickening of the mucosa. Neutrophils and lymphocytes

can also be found infiltrating the affected area (Holmes, 1985). Steel et al. (1980) estimated that 950-3000 larvae of *T. colubriformis* per week produced impairment in wool and growth in sheep. An estimation of over 1000 *T. calcaratus* produced fatal infestations in rabbits (Sarles, 1934) and ca. double this amount of worms are necessary to induce clinical disease in a one-year-old sheep (Soulsby, 1968). Moreover, it has been suggested that, in rabbits, *T. retortaeformis* affects litter survival as parasitized females have decreased milk production and consequently, smaller kittens with reduced chances of survival (Dunsmore, 1981). Body weights of rabbits and snowshoe hares *Lepus americanus* have also been impacted by *T. retortaeformis*, presumably due to villar atrophy and reduced absorptive capacity in the small intestine (Barker and Ford, 1975; Jacobson et al., 1978). Iason and Boag (1988), however, determined that neither body weight or reproductive performances were affected by high infestations of this nematode when parasitizing mountain hares *Lepus timidus*. On the other hand, experiments conducted with female mountain hares showed that treating *T. retortaeformis* infections increased fecundity (Newey and Thirgood, 2004).

1.5 Immunity

Generally, helminth parasites induce a Type 2 immune response in the host, which is characterized by both Thelper type 2 lymphocytes (with production of some specific interleukins, followed by a large production of eosinophils and goblet cells hyperplasia); and later, a more effective adaptive response which, through various mechanisms (i.e. class switched immune-globulins and activated leukocytes), will lead to the dislodgement of the parasites and their consequent elimination (Maizels et al., 2012). Immune response against parasitic nematodes can either be displayed against specific stages of the parasite or be directed to both larval and adult phases (Balic et al., 2000; Hein et al., 2010).

In sheep, antibodies IgE, IgG1 and IgA rise systemically and locally in the intestines with a fast proliferation of eosinophils and mast cells (Balic et al., 2000). The rise of activated mast cells associated with low worm burdens in sheep (Hein et al., 2010) might help explain how the inflammatory mechanism prevents infective larvae from establishing in the host mucosa (Meeusen, 1999).

Experimental *Cooperia oncophora* infections in cattle showed that the host's immune regulatory system suffers alterations during parasite infections and responses may vary according to host species as well as to the type of nematode involved (Li and Gasbarre, 2009). Economic losses are also due to host´s immune response in the presence of subclinical infections with gastro-intestinal nematodes. There is a re-prioritization of nutrient utilization

and diversion is towards activation of the immune process to the detriment of regular growth and production (Page et al., 2008).

Along with viruses and bacteria, strongylid nematodes have developed strategies to avoid being targeted by host antibodies. A study has demonstrated that the infective larvae of *T. colubriformis* may evade host's immune response by presenting antigenic variation. Maass et al. (2009) identified at least three different epitopic forms in this species. Another nematode parasite strategy is the secretion of immune-modulators i.e. cysteine protease inhibitors or cystatins. These products suppress T cell responses to nematode infections and, therefore, modulate host immune responses (Hartmann and Lucius, 2003).

Generally, a host age-dependence is observed and immune-competence is assumed to be built up gradually with continual exposure to infective larvae *e.g. T. retortaeformis* in rabbits (Cornell et al., 2008) and *T. colubriformis* in sheep (Dobson et al., 1990). This is dependant not on any particular age but on the force of infection reaching a peak more rapidly – at a younger age – when it is high, or reaching it more slowly – in older animals – when it is low. This pattern called the "peak shift" is very well described by Woolhouse (1998) and Cattadori et al. (2005). In an experimental infection with ovine *T. colubriformis*, Musogong et al. (2004), for example, showed that mean nematode and faecal egg counts in juvenile rabbits were substantially higher than in adult rabbits. The same pattern was found in a study conducted by Chylinski et al. (2009) using wild rabbits and naturally acquired *T. retortaeformis*. The authors showed, in addition, that nematode length decreased in older rabbits, suggesting that host's acquired immunity may influence nematode growth as well as nematode egg production. However, *G. strigosum* seemed to modulate immunity in rabbits as no decrease in worm numbers of this species could be observed in the hosts (Murphy et al., 2011). Furthermore, the ability of host modulation of *G. strigosum* may positively influence infections by *T. retortaeformis* (Lello et al., 2004).

In addition to a negative correlation between host age and parasite abundance there is, apparently, evidence that a component of "season of birth" might influence development of the host immune response in wild rabbit populations, naturally infected by *T. retortaeformis* (Cornell et al., 2008). Rabbits born in early spring, for example, had a stronger immune response as opposed to ones born in autumn, when immune abilities were considered the poorest (Cornell et al., 2008). In any case, immunity is decreased during host pregnancy and

lactation (Khan and Collins, 2004; Cattadori et al., 2005), co-infection with the myxoma virus (Cattadori et al., 2007), poor nutrition and exposure to severe stress (Sedlak et al., 2000).

1.6 Anthelmintic resistance

Sheep producers rely mainly on the routine use of broad-spectrum chemical anthelmintics to control parasitic diseases but the development of anthelmintic resistance has been reported for the majority of the anthelmintic drugs (Coles et al., 2006; Papadopoulos, 2008; Torres-Acosta et al., 2012). Despite distinct modes of action, resistant strains of parasites began to appear a few years after the introduction of each anthelmintic group, in particular within *Haemonchus contortus, Teladorsagia (Ostertagia) circumcinta* and *Trichostrongylus* spp. (Sangster and Dobson, 2002 ; Kaplan, 2004). According to Papadopoulos (2008) the mechanism for the development of anthelmintic resistance is the survival of a small number of worms during drench treatments, contaminating the pasture with resistant larvae in the subsequent generation. There is also a percentage contribution to the population by worms *in refugia*, *i.e.* ones not exposed to the drenches, but there is a selection pressure against subsequent generations of susceptible parasites if the same class of anthelmintic is reused, leading to a dominance of resistant parasites. Frequency of treatments and sub-therapeutic doses are other factors which contribute to the perpetuation of resistant heterozygous worms therefore, there is a selection of resistant lineages of parasites (Egerton et al., 1988). Goats can also develop resistant strains of parasites that may be passed to sheep, especially in common grazing areas (Jackson, 1993).

1.7 Quarantine

Quarantine at the property level constitutes an aspect of great importance in the strategic control process for coping with worms. Sheep being introduced onto a farm may be retained in sheep yards, treated with an anthelmintic, and held until viable propagules are eliminated, before the introduced sheep are released into grazing paddocks to join the existing flock (Swarnkar and Singh, 2012). Under feasible conditions, a minimum 24-36 h waiting period and treatment with an anthelmintic drug like monopantel, for example, before release into the flock of newly acquired sheep is reasonable, according to Sager et al. (2010). Combined anthelmintic therapy with quarantine is an important tactic against resistance. Some graziers never quarantine and treat the animals brought on to their farms whilst others do it properly (Coles, 1997; Morgan et al., 2012).

1.8 Host specificity

Wild animals are able to cross barriers established to protect livestock and play a role in the cross-transmission of some parasitic nematodes also common to domestic ruminants. In Sweden, for example, free-ranging deer of several species were recognized to have a potential to breach farm boundaries and share several helminth species with sheep (Nilsson, 1971; Borgsteede, 1982). Sheep are also known to share nematode parasites with goats (Torres-Acosta and Hoste, 2008), cattle, camels (Kumsa and Wossene, 2006), horses (Bucknell et al., 1995), pigs (Roberts, 1940), possums (Stankiewicz et al., 1996) and reindeer (Hrabok et al., 2006). Occasionally and incidentally, ovine nematodes have been found even in monkeys and humans (Joe, 1947; Lattès et al., 2011). Five to 15 adult nematodes of the genera *Oesophagostomum* sp, *Trichuris* sp, and the ruminant nematode *Haemonchus* sp. were retrieved from plateau pikas (*Ochotona curzoniae*) in China (Wang et al., 2009). A small number of wild hares and rabbits have been found presenting natural infections of sheep worms (Boag, 1987b; Saulai and Cabaret, 1998; Beck and Pantchev, 2009; Usai et al., 2012). Surveys reported numbers of ovine *Teladorsagia circumcincta*, *Nematodirus* spp., *Trichostrongylus axei*, *T. colubriformis* and *T. vitrinus* (Mykytowycz, 1956; Mackerras, 1958; Hesterman and Kogon, 1963; Boag, 1972, 1987a; Saulai and Cabaret, 1998). The ovine nematodes *T. colubriformis* and *Strongyloides papillosus* have been successfully established in laboratory rabbits e.g. (Sommerville, 1963; Nwaorgu and Connan, 1980; Hoste et al., 1988) and Stott *et al*. (2009) showed that the European brown hare is also permissive to *Teladorsagia circumcincta* and that *Trichostrongylus colubriformis, T. rugatus*, *T. vitrinus* and small numbers of *Nematodirus* spp. and *Cooperia* sp. have the ability to develop to the adult stage in European hares. However, the hares studied by Stott *et al*. (2009) were captive juvenile hares in a highly artificial environment and consequently may have experienced stress-induced immunosuppression, and hence the study may not represent the situation in the field.

1.9 Evolution

The sub-order Trichostrongylina is a large and well-distributed taxon of nematode parasites which were grouped together mainly by their morphological and evolutionary traits (Durette-Desset, 1985). The three super-families within this sub-order: Trichostrongyloidea, Molineoidea and Heligmosomoidea (Durette-Desset and Chabaud, 1993), present nematode parasites of lagomorphs that occur in the same sub-family (or genus in the case of *Trichostrongylus*) as those which parasitize ruminants. *Graphidium strigosum*, known to parasitize lagomorphs and *Haemonchus contortus*, which affects sheep (and, to a lesser extent cattle), both infect the gastric mucosa of their hosts. Phylogenetic studies based in morphological characters and molecular analyses have not yet elucidated, in evolutionary terms, the specific relationship between these two species, however, it appears that they probably had a common ancestor within the Trichostrongyloidea (Chilton et al., 2001; Gouÿ de Bellocq et al., 2001; Audebert et al., 2005). In terms of evolution, the most probable hypothesis is that adaptation of trichostrongyloids to lagomorphs would have occurred prior to adaptation to ruminants and that the species of parasites existent in the latter, originated from those of lagomorphs (Durette-Desset et al., 1999; Audebert and Durette-Desset, 2007). The similar herbivorous habits of Lagomorpha and Ruminantia would have exposed the evolving ruminant species to the helminths of the lagomorphs, providing the degree of contact necessary for host-switching to become possible (Chabaud, 1965; Audebert and Durette-Desset, 2007).

1.10 Distribution of lagomorphs and sheep in Australia

The distribution of the hare is over an area of south-eastern Australia occupying *c.* 700,000 km^2 (Fig. 1.1) (Jarman, 1986; Stott, 2003b) which is largely within Australia´s Mediterranean climate zone, characterized by mild humid winters and hot, dry summers. The land is mainly used for agriculture, constituted by integrated wheat and sheep grazing through much of the year on pastures and, in the summer, on crop stubbles (Stott and Harris, 2006).

In Australia, the rabbit occupies an area of 4,500,000 km^2 (Fig. 1.2) including almost the entire distribution of both the hare and the sheep (Fig. 1.3), and hence there are high levels of overlap between the distributions of the three species. In many areas, the overlap also occurs on a very fine scale, and the home ranges of hares (up to 200 ha) include areas also used by rabbits and sheep, including flocks on neighbouring farms (Stott, 2003b).

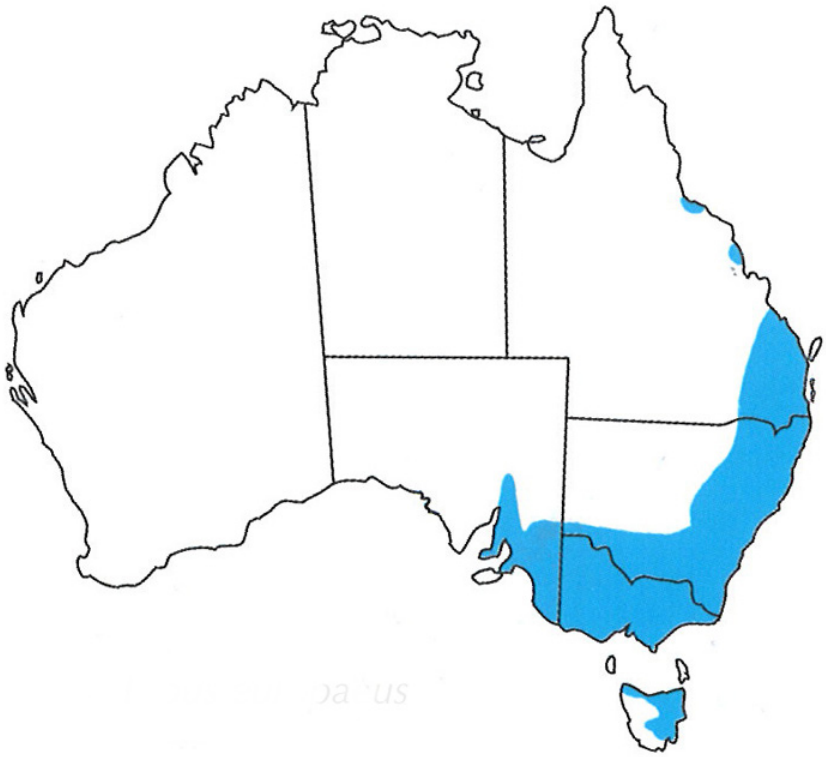

Fig.1.1 Distribution of the hare in Australia (Jarman and Stott, 2008)

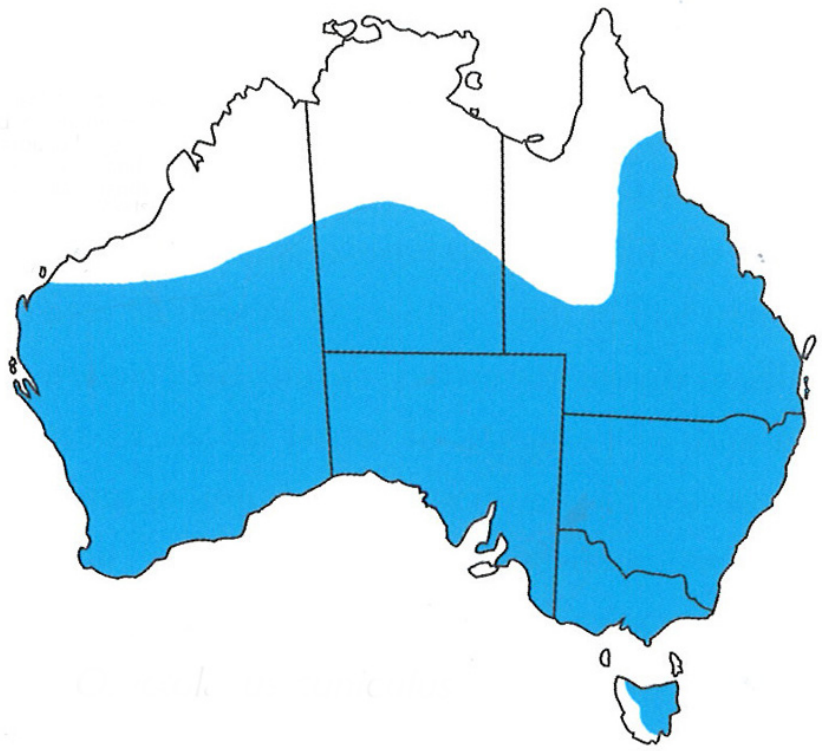

Fig. 1.2 Distribution of the rabbit in Australia (Jarman and Stott, 2008)

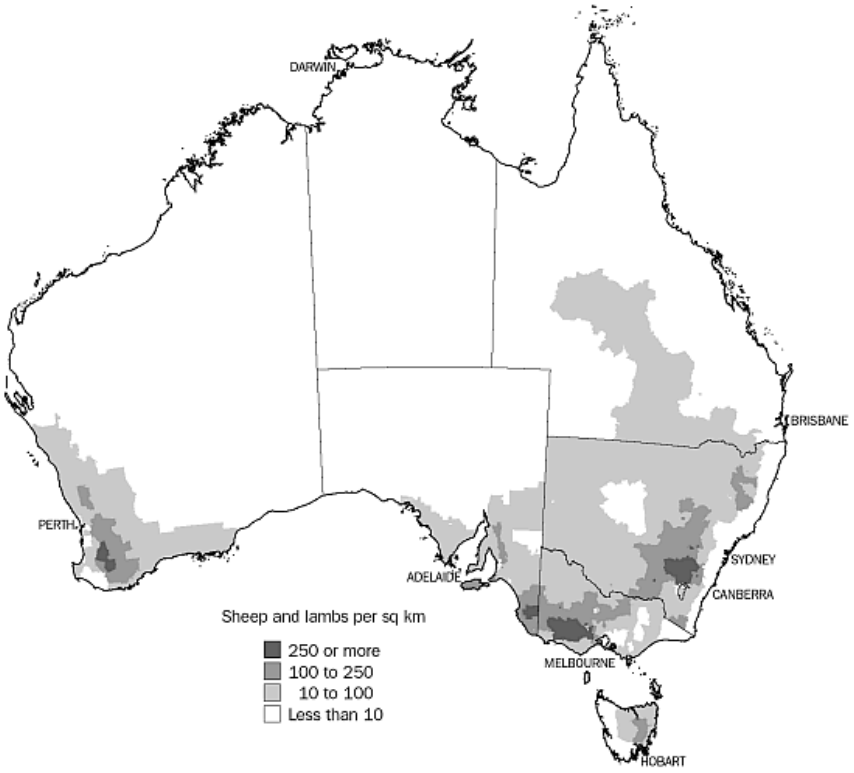

Fig.1.3. Distribution of sheep and lambs in Australia (Australian Bureau of Statistics, 2006)

1.11 Life cycle and nematode availability on pasture

The Trichostrongyloidea has a direct life cycle with a free-living stage and a parasitic stage. The duration of the free-living stage is from 3-9 days. If not equivalent, the larval and the maturation periods, as well the pre-patent period, are relatively shorter in lagomorph hosts than in ruminant hosts (Audebert and Durette-Desset, 2007). The life cycle of a trichostrongylid is illustrated below (Figure 1.4).

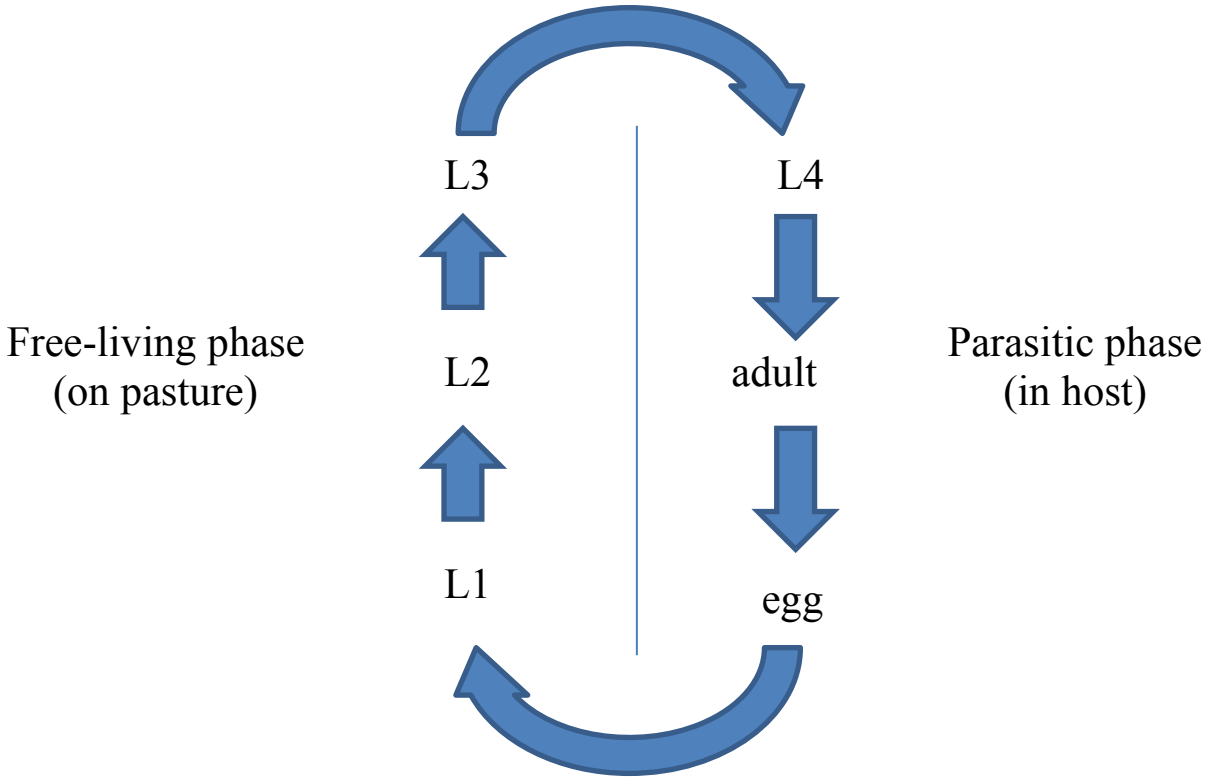

Fig. 1.4 General life cycle of a trichostrongylid nematode parasite. L1 to L4 are larval stages.

The epidemiology of the helminth parasites of sheep has been subject of many studies. In the winter rainfall areas there is a dominance of *T. circumcincta* and other *Trichostrongylus* spp. These nematodes are not as sensitive to desiccation as *H. contortus*, a worm that is prevalent in summer rainfall areas (O'Connor et al., 2006). Despite evidence suggesting that one *Trichostrongylus* spp. or another may be more prevalent in sheep in some regions rather than others (Gray and Kennedy, 1981; Beveridge and Ford, 1982), the *Trichostrongylus* spp. that commonly occur in sheep in Australia are four: *T. colubriformis*, *T. vitrinus*, *T. rugatus* and *T. axei* (Pullman et al., 1988; Beveridge et al., 1989a; Bailey et al., 2009).

Influence of the climate of a particular region is an important factor on the survival and development of the free living stages and hence a factor influencing the prevalence of each

species. Hot dry summer months are unsuitable for larval development; however, numbers of *T. circumcincta* and *Trichostrongylus* spp. may survive in faecal pellets (Young, 1983) from which they migrate onto pasture following onset of slight falls of rain. Larvae which survive in faeces over summer may lead to serious infections in the following autumn (Southcott et al., 1976). However, without rain, the emergence of infective larvae is considerably reduced. Pastures on farms can be infested for much of the year with large numbers of livestock infective larvae. Agneessens (1997) and Tessier and Dorchies (1997) have recorded densities of larvae of ruminant origin exceeding 1400 kg^{-1} dry matter (DM) and hares eat *c.* 190 g dry matter day^{-1} (Stott, 2008). Considering typical levels of infestation of sheep and their grazing hence defaecation patterns, hares are much more likely to be exposed to infective larvae of ovine origin than of lagomorph origin; and considering the proportions of ovine parasitic nematodes that are resistant to the various anthelmintics, it is inevitable that hares would be ingesting the larvae of resistant strains of nematodes. However, the fate of those larvae is unknown.

Conclusion

There is a high level of spatial overlap between sheep and hares in Australia (Stott, 2003b, 2008) and experimental infections with ruminant nematodes demonstrated susceptibility and even permissiveness of lagomorph hosts to internal parasites of sheep (Hoste and Fort, 1992; Audebert et al., 2003; Stott et al., 2009). However, it remained to be ascertained if sheep nematodes could be found, in significant numbers, in hares and in rabbits in the field under natural conditions. If this occurred, it would, perhaps, have implications for the management of the spread of resistant worms in livestock and impact hare and rabbit populations themselves (Dobson et al., 2001). Although rabbits are much less mobile than hares, and less likely to breach quarantine barriers, the role of the rabbit could be conveniently assessed during this same investigation.

The aim of this thesis was to assess if and to what extent the hare and the rabbit populations were infested with internal nematode parasites of sheep in natural situations in Australia. To fulfil the research aim, the research had the following specific objectives:

Analyse the gastrointestinal tracts of hares and rabbits obtained from the vicinity of sheep in three different regions of south-eastern Australia seeking ovine nematode parasites and quantify and identify nematode parasites to species through spicule morphology and confirm those findings with conventional PCR.

Thesis structure

In chapter 2, Morphology, I will give a brief description of the nematode parasites found in the gastro-intestinal tracts of lagomorph carcasses, based in available keys and spicule differentiation characters. Subsequently, I will describe the methodology utilized for this section of my work.

Next, in chapter 3, Molecular, I will give a brief introduction to current molecular studies performed in order to diagnose Trichostrongylid nematode infections, followed by the description of the material and methods utilized to perform PCR, with genomic DNA of nematode specimens.

Chapter 4 will present the results achieved with the means provided by chapter 2 and 3.

Finally, in chapter 5, a general discussion and conclusion will be drawn from the previous chapters.

Chapter 2

Morphology

2.1 Introduction

Traditional identification techniques are based on morphological examination of fine reproductive structures present in the caudal end of individual adult male and female nematodes. However, the most characteristic features for species identification, in Trichostrongylids, are the spicules and the bursal rays (Nagaty, 1932; Clapham, 1947; Gibbons and Khalil, 1982).

2.2 Species descriptions

2.2.1 *Graphidium strigosum* (Dujardin, 1845) Railliet and Henry, 1909

G. strigosum (Fig. 2.1) is the only species member of the genus *Graphidium* (Order Strongylida, family Trichostrongylidae, sub-family Ostertagiinae) (Massoni et al., 2011). The worms are bright red due to their blood feeding habit. The male worm is easily distinguishable from the female due to its well-developed caudal bursa. The male's body is 8-16 mm long whilst the female is 9-21 mm long (Evans, 1939; Durette-Desset and Denke, 1978). Spicules are 1.1 to 2.4 mm long (Neveu-Lemaire, 1936; Durette-Desset and Denke, 1978). The gubernaculum is about 93 μm long (Massoni et al., 2011).

Fig. 2.1 Posterior end of a male *G. strigosum*. Spicules and bursa are shown.

2.2.2 *Trichostrongylus retortaeformis* (Zeder, 1800)

Lagomorphs are also natural hosts of the gastro-intestinal nematode *T. retortaeformis* (Fig. 2.2) (Order Strongylida, family Trichostrongylidae, sub-family Trichostrongylinae). The male worm is 5-7 mm long, and the female is 6-9 mm long (Neveu-Lemaire, 1936; Levine, 1968; Soulsby, 1968). There are discrepancies between reports of spicules length: 115-158 µm long (Nagaty, 1932), 100-110 µm (Neveu-Lemaire, 1936), 120-140 µm (Mönnig, 1947; Soulsby, 1968) or 129 µm long (Clapham, 1947). The two spicules of a pair are similar in size and their distal portion is bent at an acute angle. The gubernaculum is boat shaped and 40-79 µm long (Nagaty, 1932) or 63-72 µm long (Clapham, 1947).

Fig. 2.2 Posterior end of a male *T. retortaeformis* showing spicules, gubernaculum and bursa

2.2.3 *Trichostrongylus colubriformis* (Giles, 1892)

T. colubriformis (Fig. 2.3) (Order Strongylida, family Trichostrongylidae, sub-family Trichostrongylinae) is amongst the most common nematode parasites of ruminants, especially sheep. The lengths of the male and the female worms of this species are 4-7 mm and 5-8 mm long, respectively. The spicules are long and slender, equal in size and terminate in a barb-like tip. They measure 120-171 µm. The gubernaculum has a typical boat shape and is 64-88 µm long (Nagaty, 1932; Ghasemikhah et al., 2011).

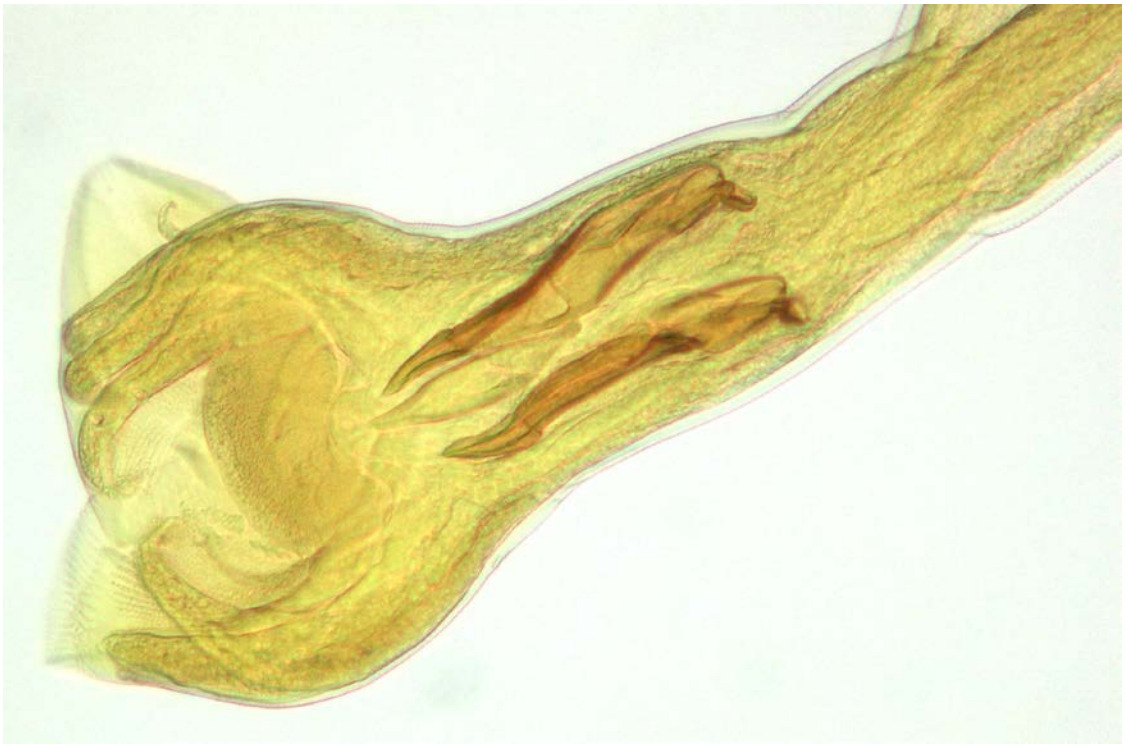

Fig. 2.3 Posterior end of male *T. colubriformis* showing spicules, gubernaculum and bursa

2.2.4 *Trichostrongylus rugatus* (Mönnig, 1925)

T. rugatus (Fig. 2.4) (Order Strongylida, family Trichostrongylidae, sub-family Trichostrongylinae) is an intestinal nematode parasite of ruminants. The male worm is 4-6 mm long and the female is 5-8 mm long. The spicules are stout, unequal in size and present characteristic creases near the caudal end. They measure 135-152 µm. The gubernaculum is 79-88 µm long (Nagaty, 1932; Neveu-Lemaire, 1936).

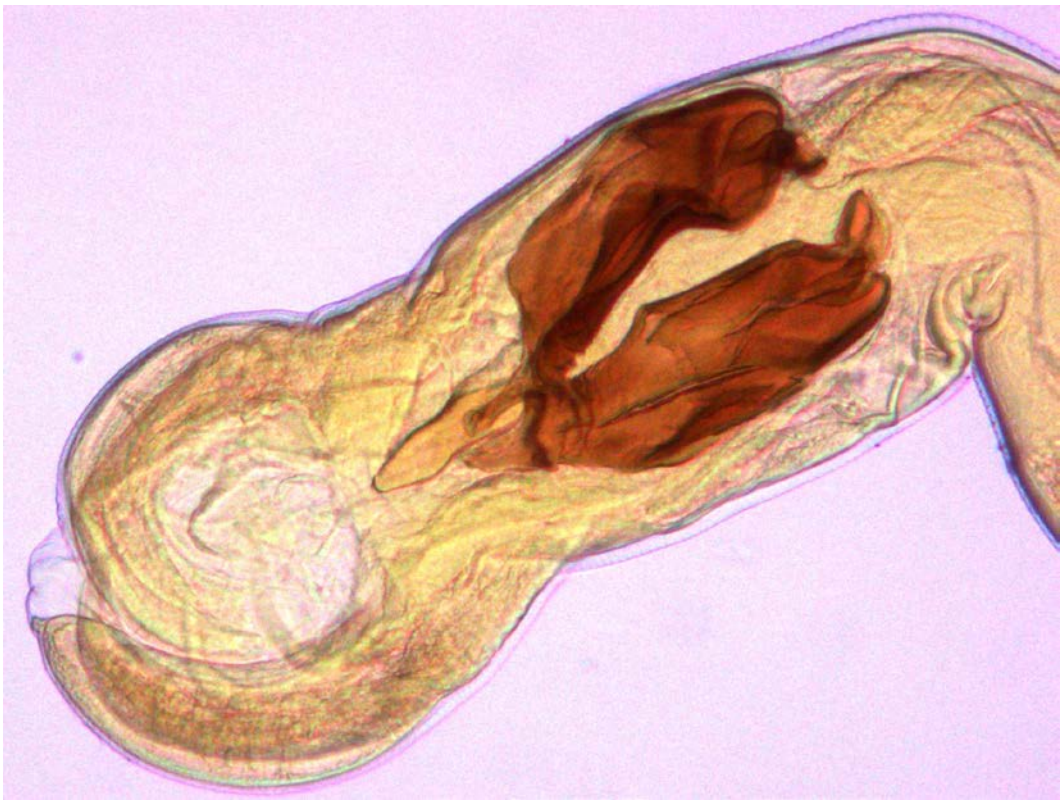

Fig. 2.4 Posterior end of a male *T. rugatus* showing spicules, gubernaculum and bursa

2.2.5 *Passalurus ambiguus* (Rudolphi, 1819)

P. ambiguus (Fig. 2.5) (Order Oxyurida, family Oxyuridae) is a stout, whitish "pinworm" that occurs in the caecum and the colon of lagomorphs worldwide (Rinaldi et al., 2007). The male is 4-5 mm long and the female is 9-11 mm long. The spicules are 90-120 μm (Soulsby, 1968).

Fig. 2.5 Male specimen of *Passalurus ambiguus* - posterior end is on the left upper corner

2.3 Methods

2.3.1 Study sites

2.3.1.1 Site selection criteria

At all sites, sheep populations were sympatric with a lagomorph population: hares, rabbits or both. Sampling sites included paddocks previously grazed by sheep or in vineyards adjacent to sheep pastures. Lagomorph carcasses were acquired from November 2010 to August 2012 from the Adelaide region of South Australia, the western district of Victoria near Hamilton (hares only) and the central western New South Wales near Wagga Wagga. Maps of sampling sites are shown in figures 2.6 and 2.7 as follows:

Fig. 2.6 Hare sampling areas in south eastern Australia. * Hare icon size is relative to sample size.

Fig. 2.7 Rabbit sampling areas in south eastern Australia. * Rabbit icon size is relative to sample size

2.3.1.2 South Australia site

The main source of hare carcasses was South Australia with 13 different sampling sites near the Adelaide region (Fig. 2.8 A-M). Most hare samples were collected in vineyards on a farm located in Langhorne Creek, 18 km from Strathalbyn. The vineyards were in the immediate vicinity of sheep pastures. Another significant sampling site was Ashbourne, 11 km from Strathalbyn which contributed 21 hares and six rabbits. Seven rabbits and three hares were taken in Williamstown. Other sheep properties contributed only one to three hares.

The grassland sampling areas of South Australia encompass two distinct climate zones near the Adelaide region, one, in the north, is characterized by hot dry summers and cold winters with a mean maximum annual temperature of 22.9°C and a minimum of 10.7°C (highest temperature is 45.6°C and lowest is 0°C); with a mean annual rainfall of 434.8 mm, whilst the other one, in the south, in the region of Strathalbyn, belong to the same climate zone of the sampling areas of temperate Victoria and New South Wales, where winters are cold and summers are only warm. The mean maximum annual temperature is 21.6°C and the minimum is 10.1°C (highest temperature is 45°C and lowest is -3°C); the mean annual rainfall is 480.3 mm and the relative humidity is 50-68%. Wet winters with low summer rainfalls are characteristic here.

2.3.1.3 Victoria site

A considerable number of hare carcasses for this study was taken from only one sampling site in Victoria, at the Department of Primary Industries (DPI), Hamilton (Fig. 2.8 N). In this area, sheep, cattle and hares were sympatric. Rabbits were only spotted near the built areas.

The elevation of the site is 200 m. The mean maximum annual temperature is 18.4°C and the minimum is 7.3°C (highest temperature is 44°C and lowest is -4.5°C); the mean annual rainfall is 662.9 mm and the relative humidity is 80%.

2.3.1.4 New South Wales site

The contribution of hare carcasses was low amongst the six sampling sites of New South Wales (Fig. 2.8 O-T). However, the main source for rabbit carcasses was a sheep farm located south of Wagga Wagga and in Weejasper. Hares were not targeted as much by shooters, as perhaps being the property owners their focus seemed to be on the high numbers of rabbits on farms.

In the New South Wales sampling sites, located between elevations of 219-390 m, the mean maximum annual temperature is 21.3°C and the minimum is 9.9°C (highest temperature is

42.5°C and lowest, -7°C); the mean annual rainfall - more uniform throughout the year than in South Australia and Victoria - is 943 mm and the relative humidity is 73%.

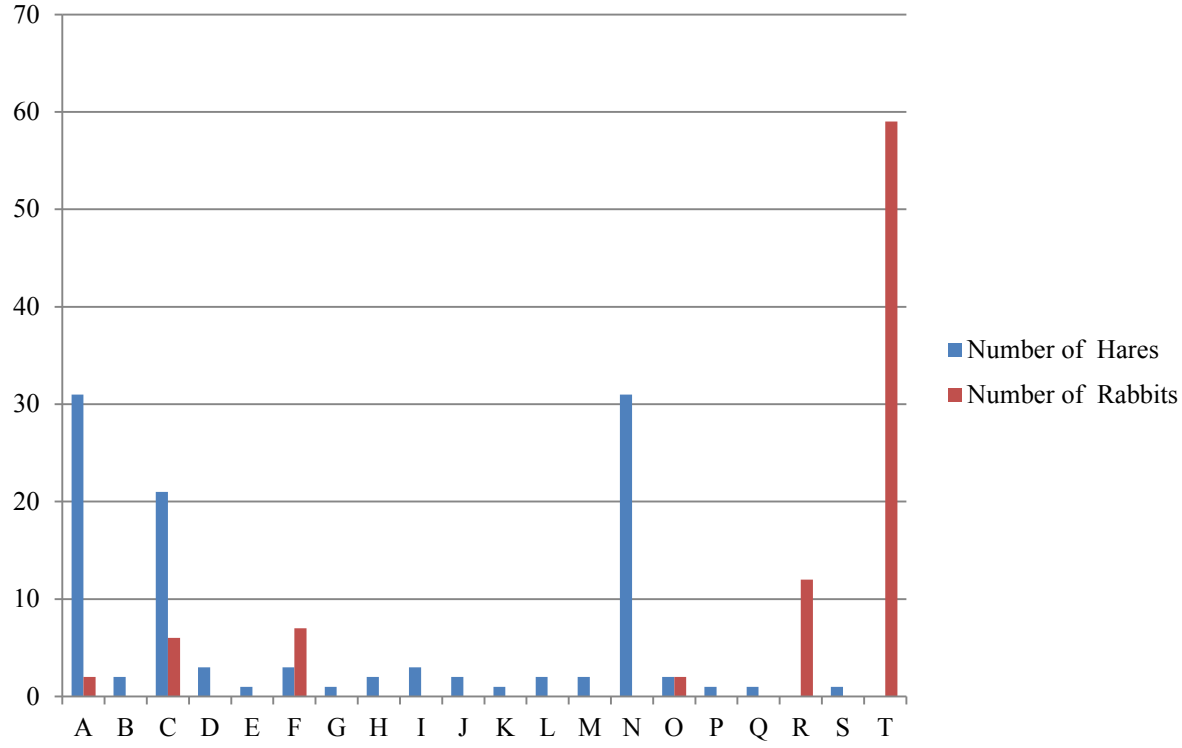

Fig. 2.8 Number of leporids acquired in each of the sampling areas in south eastern Australia

A: Langhorne Creek, SA

B: Angle Vale, SA

C: Ashbourne, SA

D: Murray Bridge airport, SA

E: South of Marrabel, SA

F: Williamstown, SA

G: Reeves Plains, SA

H: Edinburgh, SA

I: Gawler River, SA

J: Kapunda, SA

K: Angle Vale, SA

L: Ponde, SA

M: Murray River, SA

N: DPI Hamilton, VIC

O: Dunmovin, NSW

P: North of Wagga Wagga, NSW

Q: Ganmain/Coolamon road, NSW

R: Weejasper, NSW

S: Illabo, NSW

T: South of Wagga Wagga, NSW

Table 2.1 Sampling collection in each state.

	Hares	Rabbits	Total
South Australia	73	15	88
Victoria	31	0	31
New South Wales	6	73	79
Total	110	88	198

2.3.2 Animal sampling

Vehicles patrolled study sites at night and lagomorphs were located and dissoriented by spotlights, then they were shot by members of the Hunting and Conservation Branch of the Sporting Shooters' Association of Australia (SSAA). Carcasses were transported from the field to the laboratory and refrigerated until the next day.

2.3.2.1 Age of leporids

Age of animals was recorded in 104 hares and 35 rabbits. Estimations of hares and rabbits' ages were made using Stroh's method which involves palpation through the skin or direct observation to determine the degree of the distal epiphyseal thickening of the ulna. This method is useful for distinguishing between juveniles and adults. If the epiphyseal thickening was clearly palpable, the age of the leporid corresponded to a class from six to eight months old (Fig. 2.9 A) (Bujalska et al., 1965), if the structure was faintly perceptible, the animal was at about one year old (Fig. 2.9 B). If no thickening could be felt, the animal was considered to be over one year old (Fig. 2.9 C). Hares and rabbits were classified into only two categories in this study: juveniles (when animals presented clearly palpable epiphyseal ulnar thickening) and adults (when thickening was only faintly perceptible or not detectable). Leverets and kittens were not targeted in this study.

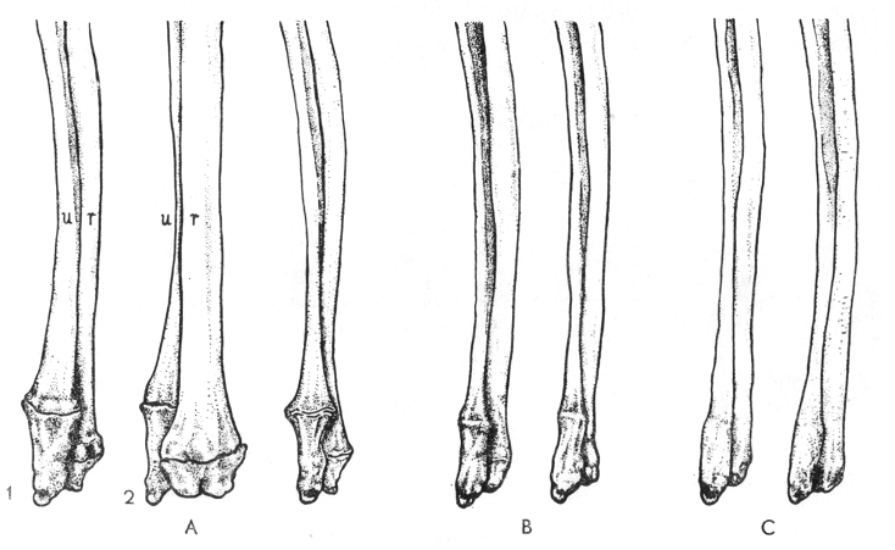

Fig. 2.9 Changes in the epiphyseal thickening of the ulna in the hare (Bujalska et al., 1965).

2.3.2.2 Gender of leporids

Sex of leporids was recorded in 109 hares and 39 rabbits.

2.3.3 Nematode sampling

The gastrointestinal tract was removed from each carcass, and the stomach, small intestine and large intestine were separated and examined for gross lesions. For each sector, the contents were washed separately and extensively with tap water, poured through wire mesh screens and finally into a jar with a final aperture of 300 µm mesh fitted into its lid to remove smaller debris, then, contents were poured into Petri dishes with one drop of parasitological iodine for observation under a dissecting microscope. Total helminth counts were performed. Adult male nematodes were separated and cut in half with a razor blade, and the posterior half was cleared with lactophenol for morphological differentiation under a stereo-microscope. The anterior halves were stored in ethanol 70% for *post priori* molecular approaches.

2.3.3.1 Spicule identification

Male nematode specimens were identified to species using spicule morphology. Spicules were observed using a compound microscope, at 20 X and 40 X magnifications, and identified to species using published identification keys (Clapham, 1947; Soulsby, 1968; Fukumoto et al., 1980; Stankiewicz et al., 1996; Sager et al., 2010).

Spicules and gubernacular lengths and widths were measured with the help of the software Analysis.

Chapter 3

Molecular assessment: Polymerase chain reactions

3.1 Nematode diagnostics

The accurate diagnosis of parasitic diseases is essential to epidemiologic and genetic studies of helminth parasites and anthelmintic efficacy (Grant, 1994). Identification of strongylid nematodes is traditionally based on morphological characteristics of mature developmental stages. However, means for the identification of immature stages (eggs or larvae) and some female worms are frequently unavailable (Soulsby, 1968; Berry et al., 2008). Furthermore, there was a need for improved illustrated identification keys (Lichtenfels et al., 1997).

Recently, many advances in the development of molecular techniques have been made for the identification of helminth parasites, particularly with the improvement of polymerase chain reaction (PCR) methods that allow exponential amplification of short DNA sequences. PCR enables rapid and reliable characterization of many different species of nematode parasites. Examples of the use of molecular approaches in veterinary parasitology are reviewed by McKeand (1999), Prichard and Tait (2001), Gasser et al. (2008) and Demeler et al. (2012).

Given the success of some recent DNA-based methods in developing assays utilizing the first and second internal transcribed spacers (ITS-1 and ITS-2), and ribosomal DNA (18S, 28S) for the identification of livestock strongylid parasites species, improvement and establishment of reliable cost-effective, sensitive and quantitative assays are promising (Bott et al., 2009). They can be soon expected to be of use not only in the laboratory context but also for specific characterization of parasitic nematodes in the field on a daily basis. Moreover, inestimable data can be provided for phylogenetic studies.

3.2 Material and methods

3.2.1 DNA extraction

The anterior ends of males and entire female adult worms (one or, grouped, up to ten animals according to availability) were fixed and stored in 70% ethanol until DNA extraction. Then, samples were washed with distilled water and phosphate buffered saline (PBS) prior to isolation of genomic DNA. Genomic DNA was isolated from nematodes either using QIAamp DNA Mini Kit (Qiagen) or with a manual protocol described as follows: In a 1.5 ml centrifuge tube, 120 µl of TE (Tris and EDTA) buffer, 9 µl sodium dodecyl sulfate 10% (SDS) and 20 µl of protein kinase 3mg/ml was added with the nematode, mixed and incubated at 55°C for two hours. Then, 150 µl of phenol chloroform was added, mixed and centrifuged for two minutes at 13,000 rpm. The clear phase was transferred to a new tube to which phenol chloroform was again added, and the tube was again centrifuged. The clear phase was transferred to another tube and 1 ml of 99% ethanol was added. The tube was incubated in the freezer for 15 minutes. Afterwards, it was centrifuged and the liquid was drained. 180 µl of TE buffer was added into the tube, which was, then, vortexed and incubated at 55°C for ten minutes. 20 µl of sodium acetate 3M was added, then, 500 µl absolute ethanol. The solution was mixed and centrifuged for one minute. The supernatant was drained and one ml of 80% ethanol was added. The sample was centrifuged for another minute and the liquid was drained. The tube was left to dry out at room temperature for 20 minutes. Finally, 30 µl of TE buffer was added and the tube was incubated at 55°C for one to two hours. Samples were vortexed and stored in the freezer until usage.

3.2.2 Primer design

The second internal transcribed spacer (ribosomal DNA) or ITS-2 gene sequences of *T. colubriformis*, *T. rugatus*, *T. vitrinus* and *T. retortaeformis* were retrieved from existing data on GenBank, accession N°s: HQ844229; AB503252; AB503251; HQ389232; EF427624; EF427622; X78066; Y14818; AY439027; X78064 to design generic *Trichostrongylus* spp. primers: 5'-TCGAATGGTCATTGTCAA-3'(forward); 5'-TAAGTTTCTTTTCCTCCGCT-3'(reverse).

3.2.3 *Conventional PCR set up*

The concentrations of $MgCl_2$ and dNTP utilized in each final 25 µL tube were of 1.5 mM and 0.2 mM, respectively. Forward and reverse primers had a final concentration of 0.4 uM each. 1 Unit of Taq DNA polymerase was utilized and 5 µL of genomic template and negative controls (distilled water with no DNA) were included in the PCR runs.

PCR was conducted in a Kyratec SC 200 thermal cycler using the following parameters: denaturation at 94°C for 45 s (38 cycles), 56°C for 45 s for annealing and 72°C for 1 min for extension. The PCR run was finalised with another final extension at 72°C for 5 min and one cooling cycle at 20°C.

Subsequently, amplicons were subjected to electrophoresis in 2% agarose gel as described by Bott (2009) and the lengths were compared to a 100 bp ladder (brand). Results of gel electrophoresis are shown in Fig. 3.1

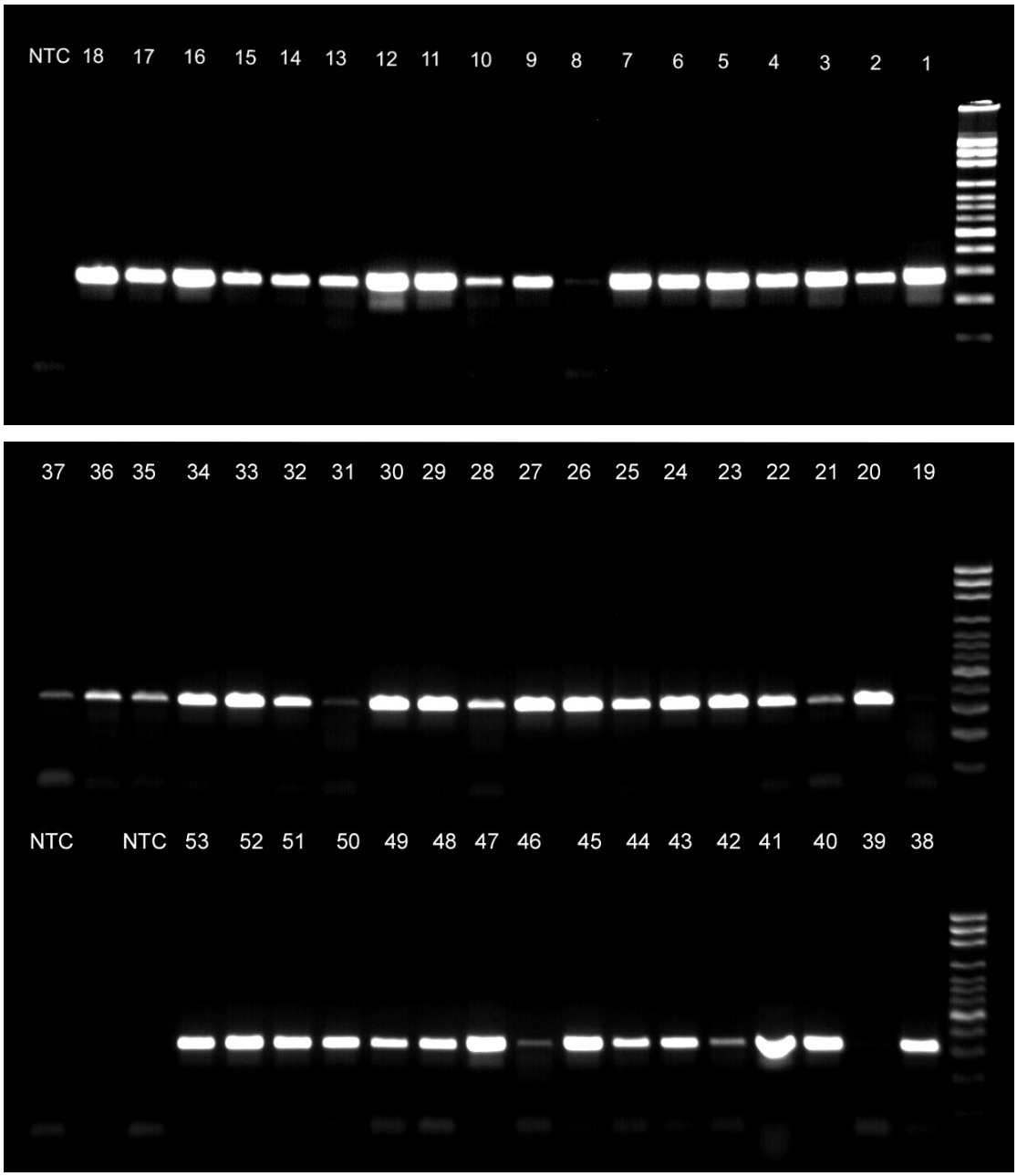

Fig. 3.1 Amplicons of c. 350 bp in agarose gel, NTC = negative control

PCR products were submitted for sequencing to the Australian Genome Research Facility Ltd., Adelaide, Australia. Sequences were aligned with BioEdit 7.1.3 and consensus sequences were analysed with Nucleotide BLAST®. 52 DNA sequences (internal transcribed spacer 2 (ITS-2), complete sequence; and 28S ribosomal RNA gene, partial sequence) of *Trichostrongylus* species found in this study were deposited in GenBank®. The accession numbers are in table 3.1.

Species	Host	Accession number
Trichostrongylus retortaeformis	Hare	JX046418.1
Trichostrongylus colubriformis	Hare	BankIt1600253 Seq1 KC521364
Trichostrongylus colubriformis	Hare	BankIt1600253 Seq2 KC521365
Trichostrongylus colubriformis	Hare	BankIt1600253 Seq3 KC521366
Trichostrongylus colubriformis	Hare	BankIt1600253 Seq4 KC521367
Trichostrongylus colubriformis	Hare	BankIt1600253 Seq5 KC521368
Trichostrongylus colubriformis	Hare	BankIt1600253 Seq6 KC521369
Trichostrongylus colubriformis	Hare	BankIt1600253 Seq7 KC521370
Trichostrongylus retortaeformis	Hare	BankIt1600253 Seq8 KC521371
Trichostrongylus colubriformis	Hare	BankIt1600253 Seq9 KC521372
Trichostrongylus colubriformis	Hare	BankIt1600253 Seq10KC521373
Trichostrongylus colubriformis	Hare	BankIt1600253 Seq11KC521374
Trichostrongylus colubriformis	Hare	BankIt1600253 Seq12KC521375
Trichostrongylus retortaeformis	Hare	BankIt1600253 Seq13KC521376
Trichostrongylus colubriformis	Hare	BankIt1600253 Seq14KC521377
Trichostrongylus colubriformis	Hare	BankIt1600253 Seq15KC521378
Trichostrongylus colubriformis	Hare	BankIt1600253 Seq16KC521379
Trichostrongylus colubriformis	Hare	BankIt1600253 Seq17KC521380
Trichostrongylus colubriformis	Hare	BankIt1600253 Seq18KC521381
Trichostrongylus colubriformis	Hare	BankIt1600253 Seq19KC521382
Trichostrongylus colubriformis	Hare	BankIt1600253 Seq20KC521383
Trichostrongylus colubriformis	Hare	BankIt1600253 Seq21KC521384
Trichostrongylus colubriformis	Hare	BankIt1600253 Seq22KC521385
Trichostrongylus colubriformis	Hare	BankIt1600253 Seq23KC521386
Trichostrongylus colubriformis	Hare	BankIt1600253 Seq24KC521387
Trichostrongylus retortaeformis	Hare	BankIt1600253 Seq25KC521388
Trichostrongylus colubriformis	Hare	BankIt1600253 Seq26KC521389
Trichostrongylus colubriformis	Hare	BankIt1600253 Seq27KC521390
Trichostrongylus retortaeformis	Hare	BankIt1600253 Seq28KC521391
Trichostrongylus retortaeformis	Hare	BankIt1600253 Seq29KC521392
Trichostrongylus colubriformis	Hare	BankIt1600253 Seq30KC521393
Trichostrongylus colubriformis	Hare	BankIt1600253 Seq31KC521394
Trichostrongylus rugatus	Hare	BankIt1600253 Seq32KC521395
Trichostrongylus rugatus	Hare	BankIt1600253 Seq33KC521396
Trichostrongylus retortaeformis	Hare	BankIt1600253 Seq34KC521397
Trichostrongylus retortaeformis	Hare	BankIt1600253 Seq35KC521398
Trichostrongylus retortaeformis	Hare	BankIt1600253 Seq36KC521399
Trichostrongylus retortaeformis	Rabbit	BankIt1600253 Seq37KC521400
Trichostrongylus retortaeformis	Rabbit	BankIt1600253 Seq38KC521401
Trichostrongylus retortaeformis	Rabbit	BankIt1600253 Seq39KC521402
Trichostrongylus retortaeformis	Rabbit	BankIt1600253 Seq40KC521403
Trichostrongylus retortaeformis	Rabbit	BankIt1600253 Seq41KC521404
Trichostrongylus retortaeformis	Rabbit	BankIt1600253 Seq42KC521405
Trichostrongylus retortaeformis	Rabbit	BankIt1600253 Seq43KC521406
Trichostrongylus retortaeformis	Rabbit	BankIt1600253 Seq44KC521407
Trichostrongylus retortaeformis	Rabbit	BankIt1600253 Seq45KC521408
Trichostrongylus retortaeformis	Rabbit	BankIt1600253 Seq46KC521409
Trichostrongylus retortaeformis	Rabbit	BankIt1600253 Seq47KC521410
Trichostrongylus retortaeformis	Rabbit	BankIt1600253 Seq48KC521411
Trichostrongylus retortaeformis	Rabbit	BankIt1600253 Seq49KC521412
Trichostrongylus colubriformis	Rabbit	BankIt1600253 Seq50KC521413
Trichostrongylus retortaeformis	Rabbit	BankIt1600253 Seq51KC521414

Table 3.1 Genbank accession numbers of sequences from Trichostrongylid species found in wild lagomorphs in Australia.

Chapter 4

Results

4.1 Morphological and molecular diagnosis

In this part of the thesis, I present the final, combined, results which were obtained both through morphological and molecular techniques as described in the previous chapters.

4.1.1 Prevalence

Sampling nights: 49 (at least); unsuccessful fieldtrips: 4.

Of the total 110 hares examined, 63 (57.3 %) presented one or more species of nematode parasites. Of the total of 88 rabbits examined, 81 (92 %) presented one or more species of nematode parasites. Five species of gastro-intestinal nematodes were recorded parasitising both hares and rabbits, amongst them, two were ovine: *T. colubriformis* and *T. rugatus*. The latter was recovered from four hares and one rabbit. This is the first report of natural infections of *T. rugatus* in wild lagomorphs. Prevalence of each worm found in lagomorphs in all three sampling regions of Australia is shown in table 4.1.

Table 4.1 Nematode parasites of lagomorphs in Australia

Species	Prevalence (%)	$\bar{x}$ ± sd worms recovered/animal	Maximum number of worms retrieved
HARES (n = 110)			
G.strigosum	1 (0.9)	1	1
T.retortaeformis	15 (13.6)	21.7 ± 47.7	184 (female adult)
T.colubriformis	36 (32.7)	19.4 ± 35.1	195 (male juvenile)
T.rugatus	4 (3.6)	7.2 ± 11.8	25 (female juvenile)
P.ambiguus	1 (0.9)	34	34
RABBITS (n = 88)			
G.strigosum	44 (50)	76.8 ± 115.6	598 (female juvenile)
T.retortaeformis	54 (61.4)	21.6 ± 39.4	215 (female juvenile)
T.colubriformis	3 (3.4)	15.7 ± 11.9	24 (female juvenile)
T.rugatus	1 (1.1)	1	1
P.ambiguus	5 (5.7)	7.4 ± 5.3	15

Considering that sampling was done in three different states in Australia, prevalence of parasitism according to region is shown in Table 4.2. No significant difference was found in worm burdens in the different regions analysed ($P > 0.05$).

Table 4.2 Regional comparison of nematode prevalence in hares in Australia with mean worms recovered per animal and standard deviation.

Hares n = 110	South Australia (n=73)		Victoria (n=31)		New South Wales (n=6)	
	Prevalence (%)	$\bar{x}$ ± sd worms recovered/animal	Prevalence (%)	$\bar{x}$ ± sd worms recovered/animal	Prevalence (%)	$\bar{x}$ ± sd worms recovered/animal
G. strigosum	1 (1.4)	1	0	0	0	0
T. colubriformis	21 (28.8)	11.9 ± 12.7	11 (35.5)	28 ± 56.8	4 (66.7)	35 ± 40.1
T. rugatus	4 (5.5)	7.2 ± 11.8	0	0	0	0
T. retortaeformis	11 (15.1)	29.1 ± 54.4	3 (9.7)	1.3 ± 0.6	1 (16.7)	1
P. ambiguus	1 (1.4)	34	0	0	0	0

Table 4.3 Regional comparison of nematode prevalence in rabbits in Australia with mean worms recovered per animal and standard deviation.

Rabbits n = 88	South Australia (n=15)		New South Wales (n=73)	
	Prevalence (%)	$\bar{x} \pm sd$ worms recovered/animal	Prevalence (%)	$\bar{x} \pm sd$ worms recovered/animal
G. strigosum	3 (20)	1.7 ± 1.1	41 (56.2)	82.3 ± 118
T. colubriformis	1 (6.7)	24	2 (2.7)	11.5 ± 13.4
T. rugatus	1 (6.7)	1	0	0
T. retortaeformis	9 (60)	29.4 ± 70.3	45 (61.6)	20 ± 30.9
P. ambiguus	4 (26.7)	5.5 ± 3.7	1 (1.4)	15

In the hare samples, 61 % of juveniles and 60 % of adults were infected by one or more nematode species. Amongst gender classes, 57% of females and 65% of males were found to be infected. In the rabbit samples, 61% of juveniles and 60% of adults were infected; 94% of females and 88% of males were infected. Different age and gender classes were, statistically, equally affected ($P > 0.05$).

Chi-Square and Fisher Exact tests showed that, in general, nematode parasite infestations were not significantly different between hares and rabbits of different regions ($P > 0.05$). However, after performing the non-parametric sign test, significant statistical difference was found in the prevalences of *T. colubriformis* and *T. retortaeformis* between the hare and rabbit populations, in South Australia. The prevalence of *T. colubriformis* was distinctly higher in hares, whereas *T. retortaeformis* occurrence was more pronounced in rabbits than in hares in ($P < 0.05$). The occurrence of ovine or lagomorph helminths only, and mixed ovine/lagomorph worms in affected hares and rabbits are presented below in Figures 4.1 and 4.2. Multiple nematode parasitic infections occurred and are shown in Figure 4.3.

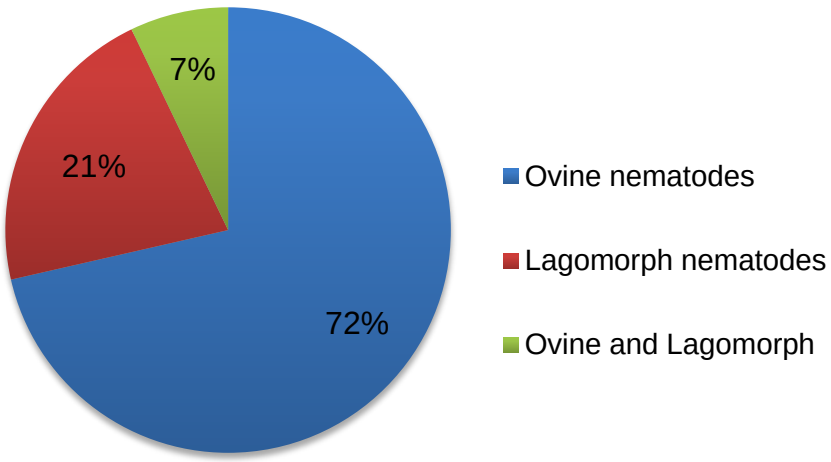

Fig.4.1 Percentage of ovine nematodes affecting parasitized hares

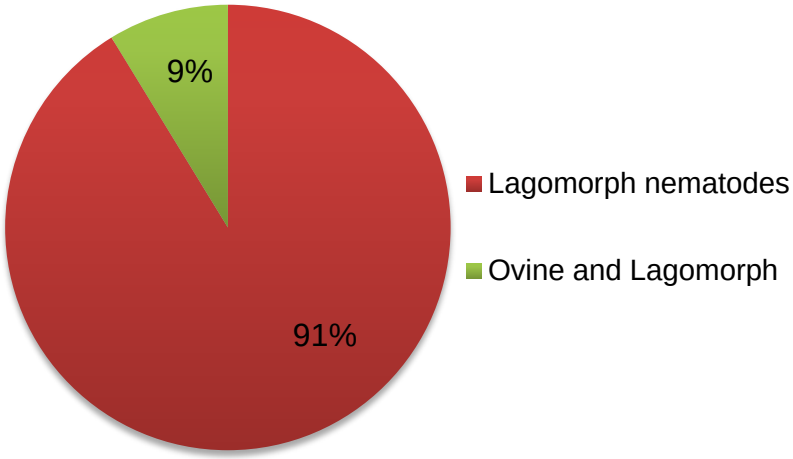

Fig. 4.2 Percentage of ovine nematodes affecting parasitized rabbits

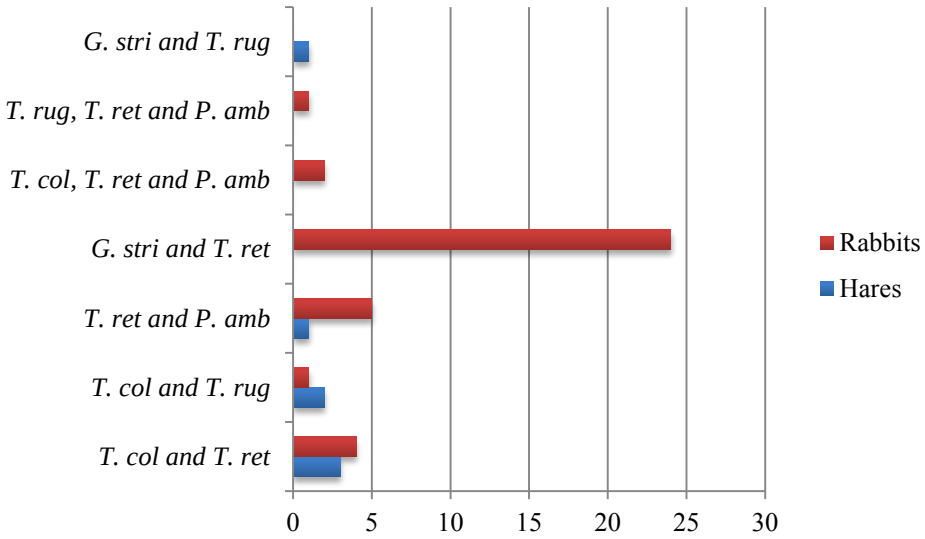

Fig. 4.3 Number of hares and rabbits with mixed nematode infections

G. stri: *Graphidium strigosum*

T. ret: *Trichostrongylus retortaeformis*

T. col: *Trichostrongylus colubriformis*

T. rug: *Trichostrongylus rugatus*

P. amb: *Passalurus ambiguus*

In order to find any relationship between the frequency of *Graphidium strigosum* and frequency of *Trichostrongylus retortaeformis* which occurred, simultaneously, in rabbits at a site in New South Wales, Spearman's rank-order correlation was performed. No significant correlation was found ($P > 0.05$). Since lagomorph carcasses were obtained in distinct seasons of the year, the impact of the latter on the prevalence of nematode parasitism on hares and rabbits was tested. There was no significant difference in prevalence of worms in the distinct seasons of the year **(**$P > 0.05$). Morphometric data of male nematodes retrieved from hares and rabbits in Australia are given in Table 4.5.

Table 4.4 Seasonal prevalence of nematode parasitism in wild lagomorphs in Australia

Hares				
Season	**Spring**	**Summer**	**Autumn**	**Winter**
Number of animals	3	16	62	29
Number affected (%)	2 (66.7)	9 (56.3)	38 (61.3)	18 (62.1)
Rabbits				
Number of animals	-	5	30	53
Number affected (%)	-	4 (80)	28 (93.3)	48 (90.5)

Table 4.5 Measurements of some male nematode structures with means and standard deviations

Species	*T.colubriformis* (n=175) $\bar{x} \pm sd$	*T.retortaeformis* (n=235) $\bar{x} \pm sd$	*T.rugatus* (n=12) $\bar{x} \pm sd$
Spicule lengths (μm)	126 ± 6.4	121 ± 13.2	129 ± 6.8
Spicule widths (μm)	22 ± 3.1	25 ± 3	42 ± 4.3
Gubernaculum lengths (μm)	68 ± 5	64 ± 6.6	74 ± 6.9

4.1.2 Necroscopic examination

Macroscopic disruptions were observed at the form of thickening of portions of the duodenal mucosa (Fig. 4.4) in a few hares affected by lagomorph-specific *T. retortaeformis*. No other visible abnormalities were detected.

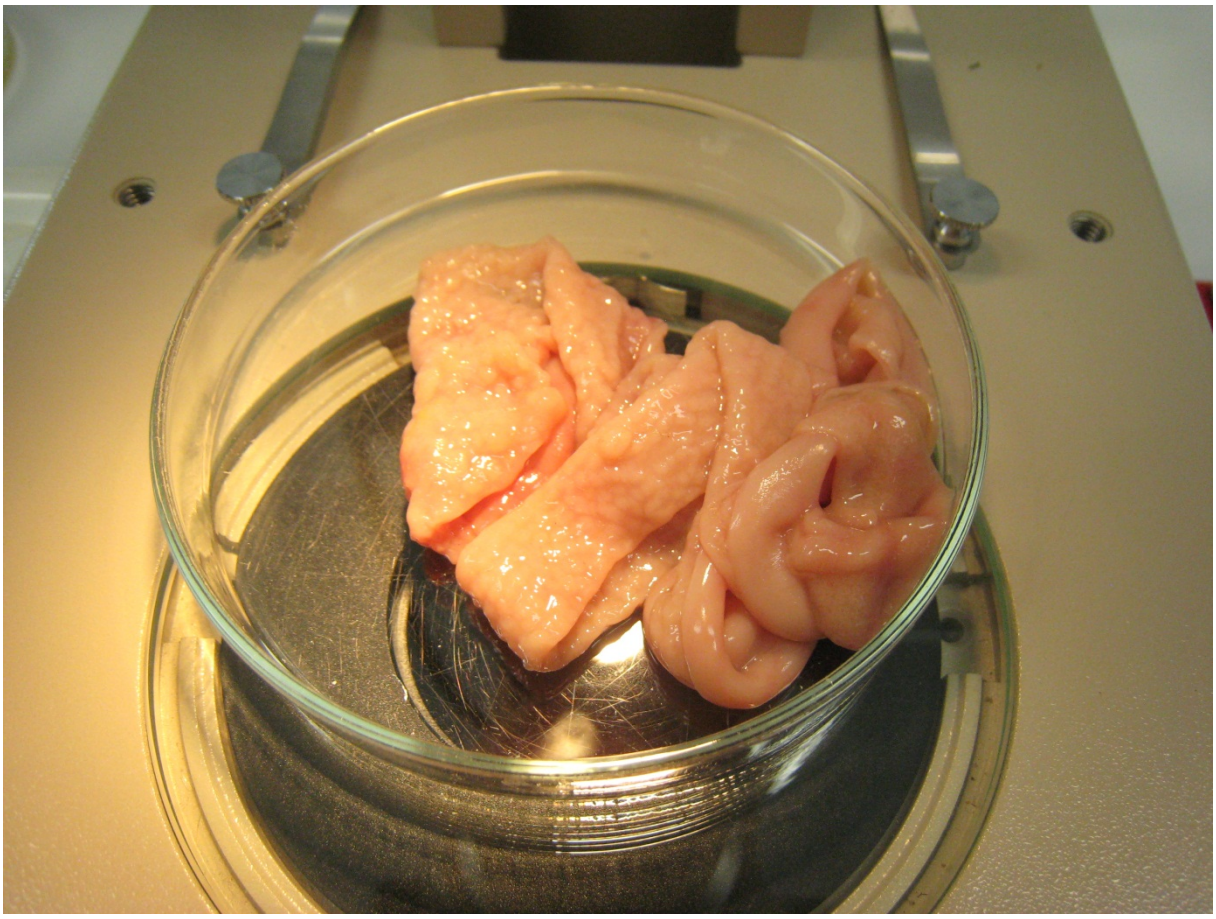

Fig. 4.4 Portion of hare duodenum presenting thickening of the mucosa

Chapter 5

Discussion

5.1 Prevalence

Natural infections of lagomorphs with ovine nematodes are confirmed in southeastern Australia. *Trichostrongylus colubriformis* and *T. rugatus* were found to be present in wild lagomorphs in the field. The two species had been established experimentally by Stott et al. (2009) and were amongst other nematodes which were able to reach adulthood in the abnormal hosts. Even though *Teladorsagia (Ostertagia) circumcincta* and smaller numbers of *Nematodirus* spp. and *Cooperia* sp. were retrieved from hares in the experiment conducted by Stott et al. (2009), and *Cooperia* spp. and *O. circumcincta* were established, in small numbers, in experimental infections of rabbits (Wood and Hansen, 1960), these species were not found in the field in the present study. The common lagomorph-specific nematode parasite *Trichostrongylus retortaeformis* was present and was equivalent in prevalence in both lagomorph species (Table 4.1), however, the lagomorph-specific stomach worm *Graphidium strigosum* had relatively high prevalence (50%) in rabbit stomachs (Table 4.1) which had the same provenance: a farm located in the central western New South Wales. A prevalence of 33% of rabbits infected with *G. strigosum* has been recorded in Australia in restricted areas (Mykytowycz, 1956). The nematode has not been found in semi-arid areas or in sub-tropical sites and only a few numbers were present at a Mediterranean zone site (Dunsmore and Dudzinski, 1968). In accordance with the previous studies, the only hare which was found to be infected with *G. strigosum*, in the present study, was from a South Australia site, which has a Mediterranean climate. According to Broekhuizen and Kemmers (1976), hares are infected by *G. strigosum* only when rabbits are present and this occurrence is restricted to milder areas. This is in accordance with the present work. The nematode *G. strigosum* largely infested rabbits in a single farm located in central western New South Wales. In this region, climate conditions are more favourable as rainfall events are more frequent and persistent (average annual rainfall between 500-600 mm), even when temperatures are high and evaporation is fast, contributing to the ability of nematode larval stages to survive desiccation and migrate on pasture (Broekhuizen and Kemmers, 1976). Unfortunately, although sympatric hares were present, hare carcasses were not obtained from this same area and assessment of presence of *G. strigosum* in sympatric hares was not made possible. Nevertheless, in the Australian Capital Territory, 6% of hares sympatric with *G. strigosum*-

infested rabbits have been reported with this same parasite, with an average of 50 worms and a maximum number of 250 nematodes retrieved from one individual hare (Hesterman and Kogon, 1963).

In general, in comparison with other lagomorph populations, the hares analysed here had low worm burdens. Equivalent nematode parasite burdens in hares were present in studies conducted by Bordes et al. (2007) and Dubinsky et al. (2010). However, no ovine worms were recorded in either of those studies. It is possible, nonetheless, that in those studies - amongst others in literature – that any ovine *Trichostrongylus* could have been mistaken for *T. retortaeformis*, as this nematode is typically found in lagomorphs, and because all *Trichostrongylus* spp. have similar sizes and the same "hair-like" appearances. Moreover, the methodology recorded for those studies does not state detailed spicule morphology was used to distinguish between the different *Trichostrongylus* spp.

5.2 Nematode species distribution

Factors including seasonal availability of free living stages and their survival on pasture in different geographic conditions (Beveridge et al., 1989a) influence the different levels of infestation of sheep by Trichostrongylids as demonstrated by Bailey et al. (2009). In 1982, Beveridge and Ford indicated that, in general, in South Australia, *T. vitrinus* was the most prevalent *Trichostrongylus* sp. in sheep and that in terms of predominance, this nematode species seemed to be more marked in more humid areas of the state. Conversely, *T. rugatus* was scarce in the same areas, but abundant in the drier and hotter areas in the northern regions of the state. According to the authors, *T. colubriformis* had a relatively constant prevalence in sheep in South Australia. However, it was not the predominant species in any particular area. Within the *Trichostrongylus* spp. of my study, *T. colubriformis* was the most prevalent species in hares for all regions examined. *T. rugatus*, a common sheep parasite considered less pathogenic than *T. vitrinus* and *T. colubriformis*, had a very modest contribution in lagomorphs, and not precisely occurring in the drier areas of South Australia. Despite my conjectures based in the work of Beveridge and Ford (1982) and that *T. vitrinus* had been found in rabbits in Australia (Roberts, 1935), this species which is considered the most pathogenic *Trichostrongylus* species to sheep (Beveridge et al., 1989b) was absent in the hares and rabbits analysed in my work. Additional abattoir studies could have determined prevalence of *T. vitrinus* and other nematode species in sheep sympatric with lagomorphs. As to the oxyurid lagomorph worm *Passalurus ambiguus*, only six animals were affected by this parasite. Its prevalence in both hares and rabbits was much lower than reported by other surveys (Boag, 1985; Allan et al., 1999; Foronda et al., 2003; Ashmawy et al., 2010).

Distribution of various nematodes in the rabbit were studied by Boag *et al.* (2001). In their study, *G. strigosum* infections increased with host age, whilst *T. retortaeformis* and *P. ambiguus* infections decreased in adult animals. In this study, age and sex did not seem to influence nematode parasitism in the hosts ($P > 0.05$), neither did different geographical localities (Table 4.2) or distinct seasons within Australia (Table 4.4). The power of the statistical tests was, however, diminished by the large variability within the classes and because of over-dispersion of parasites within host species. Interestingly, the maximum number of worms retrieved in this work was highest in juvenile animals (Table 4.1) suggesting that their acquired immunity against helminths was, presumably, not fully developed (Dobson et al., 1990; Musongong et al., 2004; Cornell et al., 2008). Leverets and kittens, which were not included in the present study, commonly present a level of immune protection transferred via maternal milk (Boag and Garson, 1993).

Ovine nematodes seemed to have a pronounced appearance amongst free-living lagomorphs in Australia with *T. colubriformis* being significantly more prevalent in hares (Table 4.1). Unlike the hares, the rabbits were only affected by sheep nematodes when lagomorph worms were also present, and this occurred in only three leporids (Figure 4.2). Hares were more commonly infected by ruminant nematodes than by lagomorph-specific nematodes (Figure 4.1). The high mobility of hares could be an explanation why they were more infected by ovine nematodes than were the rabbits. Normal levels of worm infestation in sheep, their grazing and hence defaecation patterns on farms (White and Hall, 1998), and the home ranges (Stott, 2003b), densities, and digesta throughput (Stott, 2008), of both hares and rabbits, show that hares would be much more likely to be exposed to infective larvae of ovine origin than of lagomorph origin in the sheep zone of south eastern Australia. Conversely, rabbits have a concentrated grazing pattern which would considerably increase the probability of ingesting larvae originating from rabbit faeces. Differences in immune responses between hosts may also be considered. The host-parasite relationship is complex and development of host immune response against nematode parasitism is influenced by a number of factors including host genotype, physiologic and genetic variations of parasite species (Hein et al., 2010). It is not yet clear if the two lagomorph species would harbour same levels of ovine nematodes if they were both equally exposed to infective larvae on pasture. This would require further investigations. In China, more than a hundred eggs per gram of faeces of *Haemonchus* sp. – *H. contortus* has interestingly presented cross-antigenicity with *G. strigosum* (Cuquerella and Alunda, 2009) – retrieved from naturally-infected pikas (Wang et al., 2009). In laboratory rabbits, *H. contortus* infections were not very well established since only a few adult worms

and no eggs were retrieved (Hutchinson and Slocombe, 1976; Mapes and Gallie, 1977). There is still question if the hare would follow the same pattern as the rabbit or as the non-leporid lagomorph pika. In any case, permissiveness in the hare and in the rabbit is not equal, as indicated by studies which show higher susceptibility of hares to toxoplasmosis and to *G. strigosum* infections (Broekhuizen and Kemmers, 1976; Sedlak et al., 2000).

5.3 Evolution

In terms of evolution, it is known that the three super-families within the sub-order Trichostrongylina: Trichostrongyloidea, Molineoidea and Heligmosomoidea (Durette-Desset and Chabaud, 1993) include nematode parasites of lagomorphs that occur in the same sub-family (or genus in the case of *Trichostrongylus*) as those which parasitize ruminants. Durette-Desset et al. (1999) suggested that the Trichostrongylinae would have adapted to ancient ruminants from early lagomorphs, probably during the Miocene period. However, if the premise is that ancient lagomorphs might have passed on primitive strongyles to early ruminants during the Miocene, then, it would be likely that lagomorphs (hares, rabbits and pikas) and ruminants (*e.g.* sheep, goats and even cows), shared, equivalently, trichostrongylid nematodes today. My findings show that only hares and sheep seem to have a strong natural transference of these nematode parasites. My work suggests that the transference of trichostrongylids from Lagomorpha to Ruminantia would have taken place more recently than the early Miocene. This interchange would have occurred after the radiation of both the Leporids and the Bovids. Moreover, as goats appear to have the same level of permissiveness to ovine trichostrongylid nematode parasites as sheep, perhaps host transference would have occurred in the Pliocene, after the rise of the tribe Caprini which is composed of both genera *Capra* and *Ovis* (Hassanin and Douzery, 1999).

5.4 Clinical disease

It was apparent that the nematode parasites retrieved from the carcasses were not in sufficient numbers to cause overt clinical illness in the hosts. Macroscopic disruptions of the intestinal mucosa were only observed as the form of thickened nodules (Figure 4.4) in a few hares affected by *T. retortaeformis*. Dissection of these nodules revealed more presence of *T. retortaeformis*. Migration of infective stage of this nematode (and of *G. strigosum*) to the gastro-intestinal tissue has been recorded in the rabbit, as a defence mechanism of this parasite, when conditions are adverse in the lumen (Van Kuren et al., 2013). Future histo-

pathological studies will be useful in order to understand the parasite dynamics within the host.

There were no macroscopic disruptions in the hare stomach which harboured *G. strigosum*, presumably, because of the low burden – only one worm was retrieved. Infected rabbits presented moderate infestations of *G. strigosum*, however, there was no apparent clinical disease. This is in accordance with Nickel and Haupt (1986) and Cuquerella and Alunda (2009) who did not find significant alterations, even in haematological parameters; and with Broekhuizen and Kemmers (1976), who suggested that rabbits do not show signs of gastric mucosal disruptions even with high worm infestations of these parasites. Hares, on the contrary, are less tolerant to *G. strigosum* infections and may display hyperaemia and oedema in the stomach wall (Broekhuizen and Kemmers, 1976).

5.5 Spicule Morphology

Initially, *T. retortaeformis* and *T. colubriformis* may look very similar even under microscopy. Measurements available in literature can be misleading since lengths and widths of spicules and of gubernaculums overlap many times in both species. Overall, I found that spicules of *T. colubriformis* have a more slender shape than the spicules of *T. retortaeformis*. Moreover, the caudal ends of the spicules of the latter are relatively shorter, rounder and destitute of something that resembles a corona (arrows in Figure 5.1). Nagaty (1932) describes this corona as a precise "hook-like" process of the spicule of *T. colubriformis*, which can be best visualized from its side.

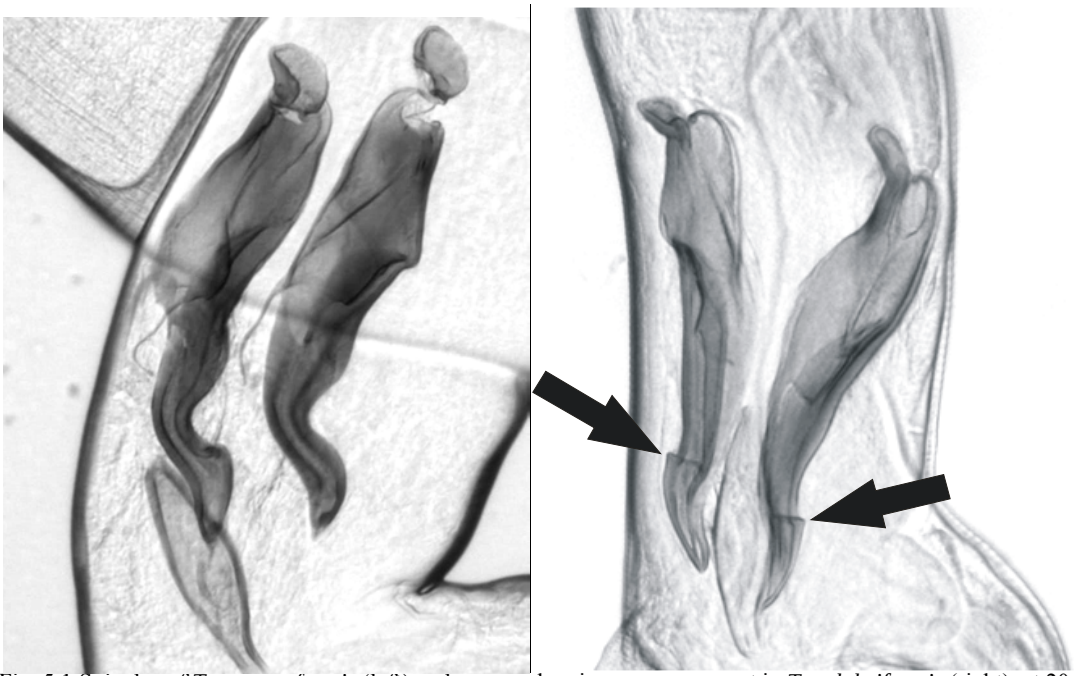

Fig. 5.1 Spicules of *T. retortaeformis* (left) and arrows showing corona present in *T .colubriformis* (right), at 20 x magnification.

5.6 Morphometry

Measurements taken from male copulatory structures of *T. retortaeformis* and *T. colubriformis*, in my study, are in accordance with the literature. However, *T. rugatus* spicular and gubernacular lengths were slightly shorter than the records found in the literature. It is conceivable that the abnormal host environment induced an atypical growth of the nematode caudal end structures (Audebert and Durette-Desset, 2007).

5.7 Molecular assay

Design of generic primers utilizing ITS-2 sequences of distinct *Trichostrongylus* spp., and subsequent PCR to detect the different species was an efficient diagnostic tool, given that the ITS-2 sequences contain homologous characters that are preserved between species (Hoste et al., 1995; Chilton et al., 1998).

It is relevant to say that in this work, male nematodes as well as female nematodes were sequenced - the male nematodes that were identified through spicule morphological techniques had their species confirmed, and female worms that could not be diagnosed via morphological methods were identified after PCR and sequencing. The sequence data deposited in Genbank may contribute to future studies on phylogenetic relationships of parasitic nematodes.

An interesting next molecular step would be the design of a real time PCR with a melting curve analysis assay. This has been proven to be more sensitive and specific, therefore, also more accurate in the determination of the different species of nematode parasites (Gasser et al., 2008; Bott et al., 2009).

5.8 Permissiveness and anthelmintic resistance

Trichostrongylus axei is a "generalist" nematode parasite, infecting small ruminants, cattle, horses, pigs and wild ungulates (Nickel and Haupt, 1986) as well as having the capacity of freely recombinating across host species (Archie and Ezenwa, 2011). Although high genetic diversity and population abundance found in this species has been associated with a fast development of anthelmintic resistance (Kaplan, 2004; Wang et al., 2009), *T. axei* presents a lower selection for resistance in comparison with other *Trichostrongylus* spp.. Archie and Ezenwa (2011) explained that *T. axei* is more commonly found in wildlife than in livestock and that when gene flow is directed from wildlife towards domesticated hosts, this might decrease selection for development of resistance in the nematode species. On the other hand, *T. colubriformis*, frequently found in hares in the present study, is much more common in sheep than in wildlife, and its rate of development of resistance is recognized to be one of the highest amongst the gastro-intestinal nematode parasites of sheep (Kaplan, 2004). Therefore, it is pertinent to raise the concern that hares may be a source of resistant strains of ovine nematode species transported between sheep farms.

5.9 Future directions

Until now, hares were an unrecognized component influencing the transmission of livestock nematode parasites to sheep and the rate of development of anthelmintic resistance between farms. Whether the European hare is actually transmitting resistant strains of ruminant nematodes to sheep or, perhaps, favouring retardation of development of drug resistance by sustaining worms *in refugia*, there is no doubt that its role should be examined more closely. More studies and experimentation should be undertaken, perhaps with the use of genetic markers, to clarify this issue. Furthermore, livestock nematode parasites in free-living hares might be of conservation significance since these worms can be associated with factors impacting the decline of populations of wild hares in many European countries. If the hare is proven to negatively influence transmission and increase rate of development of anthelmintic resistance amongst sheep farms, then nematode parasite control and management programs would, perhaps, integrate options such as hare proof fencing into their practices, along with other chemical and non-chemical strategies.

In conclusion, the results of my work suggest that hares may adversely impact control attempts of nematode parasites of sheep in south eastern Australia, however; more scientific knowledge is required in order to understand this interaction and to recommend targeted strategies (i.e. hare proof fencing or hare culling). In a broader perspective, nowadays, with the increase of interspecific interactions between wildlife, livestock and humans, transmission of diseases amongst them is not uncommon and a good management of animal parasitic diseases should consider all aspects involved.

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