Chapter 1 Introduction

Trichostrongylids (Family Trichostrongylidae) are the most important parasitic worms infecting small ruminants. Infection commonly occurs in the abomasum by *Haemonchus contortus*, *Teladorsagia* (*Ostertagia) circumcincta*, *Trichostrongylus axei*, and in the small intestine by *Trichostrongylus colubriformis*, *T. vitrinus* and *Cooperia curtecei*. In the tropics, sub-tropics and temperate areas with summer dominant-rainfall areas, infection by *Haemonchus contortus* and *Trichostrongylus colubriformis* have had a major impact on the livestock industry through effects on productivity and mortality. In a tropical country like Malaysia, worm infection in small ruminants has been identified as the second highest cause of mortality following pneumonic pasteurellosis (Sani and Rajamanickam, 1990). Economic cost (deaths, treatment costs and condemnation in abattoirs) in goats due to parasitism was estimated at RM 44,400 per annum (USD 12,700) (Fadzil, 1977) and is reportedly increasing (Sani *et al.*, 2004). In a temperate country like Australia, worm infection has been estimated to cost as much as AUD $369 million per annum (treatment cost and production loss) (Sackett *et al.*, 2006).

Economic losses due to GIN infection have continued despite heavy reliance by farmers on anthelmintic drugs, which has led to the development of GIN resistant to the anthelmintic classes. In order to reduce the dependence on anthelmintics for worm control, Integrated Parasite Management (IPM) was proposed (Walkden-Brown *et al.*, 2004), where a range of chemical and non-chemical methods are brought together to tackle GIN. Pasture management is a large component of IPM as GIN are known to spend part of their life-cycle on pastures. Understanding of the ecology of major GIN is essential to inform better IPM.

There is a good understanding of the effects of temperature on the development to L3 but understanding the effects of moisture is incomplete. Moisture available to the faecal pellet is a complex factor with contributions from various sources such as rainfall, dew and soil

and these combine with drying factors to modulate the microenvironment of faecal pellets and development success.

Key areas regarding the effect of moisture on the development of *H. contortus* and *T. colubriformis* to the infective third stage larvae (L3) remain to be elucidated and these informed the experiments reported in this thesis. The general hypothesis for these experiments was that increasing moisture will be positively associated with increasing L3 recovery for both *H. contortus* and *T. colubriformis* on day 14 relative to faecal deposition. Research questions for these experiments are;

1. Does rainfall which occurred on days before faecal deposition influence development of *H. contortus* and *T. colubriformis* to L3?
2. Does soil moisture change the sensitivity to rainfall events of *H. contortus* and *T. colubriformis* development to L3? In other words do soil moisture and rainfall amount interact to regulate L3 development?
3. Is FM suitable to integrate moisture effects and allow prediction of the development of *H. contortus* and *T. colubriformis* to L3?
4. Will the moisture factors used to predict development of *H. contortus* and *T. colubriformis* L3 apply when determining translation to grazing animals in the field?

Chapter 2 Literature review

2.1 Introduction

Sheep parasitic gastro-intestinal nematodes (GIN) from the superfamily Trichostrongyloidea are important to small ruminant production systems as infection by these GIN often leads to production loss and in severe cases will cause mortality. The most important species in this family include *Haemonchus contortus, Trichostrongylus colubriformis*, *Trichostrongylus axei, Trichostrongylus vitrinus, Ostertagia (Teladorsagia) circumcincta* and *Oesophagostomum columbianum*. These GIN spend part of their life in the host (parasitic phase) and the other part in the environment (free-living phase). In the parasitic phase, the body temperature of the host fluctuates little and environmental variables have no simple or direct effect on GIN. However in the free-living phase, these GIN are vulnerable to the effects of environmental variables (Levine, 1978). Their developmental success to third stage infective larvae (L3) and their survival on pasture during the free-living phase depend on environmental variables, mainly temperature and moisture (Thomas, 1982; O'Connor *et al.*, 2006). In this thesis, focus is given to *H. contortus* and *T. colubriformis* due to the fact both species are of great importance in Malaysia (the author's country) and the northern tablelands of NSW, Australia (the author's place of research) for small ruminant production.

In a tropical country like Malaysia, with warm temperatures and high annual rainfall, development of GIN to L3 is rapid throughout the year with 26-66% of eggs deposited on pasture successfully hatched to L3 in 9 days (Cheah and Rajamanickam, 1997), making the use of ecology-based control strategies less complicated than those in temperate countries. For example, application of rotational grazing to exploit parasite ecology in the tropics requires animals to spend less than 3-4 days grazing in each paddock followed by absence of grazing for at least 30 days (Barger *et al.*, 1994). In non-tropical, summer-rainfall areas where rainfall is not as regular and there is greater fluctuation in temperature,

development of eggs to L3 is less assured and it is more complex to use environmental variables to predict parasite ecology and hence the magnitude of the L3 population on pasture.

Models that predict GIN L3 on pasture based on temperature and moisture have been developed for *H. contortus* (e.g. Barger *et al.*, 1974) and *T. colubriformis* (e.g. Barnes *et al.*, 1988) – but only few have been fully validated in the field with grazing animals. Validation can be complex as there are many factors that can regulate temperature and moisture at the microenvironment of the GIN eggs (i.e. in the faecal pellet). Information on the moisture requirements for the successful development of *H. contortus* and *T. colubriformis* to L3 is less available than the information regarding temperature requirements. Uncertainty over moisture requirements further limits the ability to predict L3 population size on pasture. This is an area that requires further investigation. One of the most obvious and easily recorded regulators of moisture is rainfall. However other factors that modulate moisture such as timing of rainfall in relation to faecal deposition, evaporation rate and soil moisture content also require further investigation for a better and integrated understanding of how these factors regulate the developmental success of *H. contortus* and *T. colubriformis* to L3 on pasture.

2.2 Distribution of *Haemonchus contortus* and *Trichostrongylus colubriformis*

Distribution of *H. contortus* and *T. colubriformis* is influenced by climatic conditions as these species respond differently to temperature and the availability of moisture.

2.2.1 *H. contortus*

H. contortus (barber's pole worm) is a blood-sucking abomasal nematode responsible for extensive productivity loss in sheep in tropical, subtropical (Urquhart *et al.*, 1996) and summer-dominant rainfall areas (Pullar, 1953; O'Connor *et al.*, 2006): occasional effects outside these areas are linked to unseasonal weather patterns. This parasite is mostly found in the areas located close to the tropics and can be commonly found in warm and humid areas located between 30 °N and 30 °S (Urquhart *et al.*, 1996), but it can also be an important parasite outside of these regions wherever periods of warmth and moisture coincide. *H. contortus* is able to undergo hypobiosis, a prolonged but temporary arrested larval development at the fourth stage larvae (L4) in autumn. This condition is an adaptation to ensure survival of the parasite during periods of environmental adversity when conditions for translation are poor and survival of free living forms may be minimal (Blitz and Gibbs, 1972a; Gibbs, 1982). Development of the arrested L4 recommences in spring and this could contribute to the sudden rise of egg production or 'spring rise' (Blitz and Gibbs, 1972b) in sheep. Due to its ability to undergo hypobiosis, *H. contortus* is also becoming more prevalent in northern hemisphere countries with cold winters like Sweden, France, Denmark and the Netherlands (Waller *et al.*, 2004).

2.2.2 *T. colubriformis*

T. colubriformis (black scour worm) is a small-intestinal parasite and one of the most important causes of parasitic gastroenteritis in ruminants commonly found in sub-tropical and temperate summer-dominant rainfall areas (Southcott *et al.*, 1976; Bailey *et al.*, 2009b). It is also common in winter-rainfall areas (Dunn, 1978) and is widely distributed throughout the world due to its ability to tolerate cold temperature and withstand desiccation (Dunn, 1978). *T. colubriformis* is more common in warmer climates when compared to other *Trichostrongylus* species, such as *T. vitrinus* and *T. axei*.

2.3 General life cycle

H. contortus and *T. colubriformis* share the same direct life cycle. Female and male adults mate inside the abomasum (for *H. contortus*) or small intestine (for *T. colubriformis*) of host-animals and the life cycle begins with the adult female producing eggs that are passed from the gut to the pasture inside faecal pellets. This is the beginning of the free-living phase, during which the development of the eggs to L3 on pasture is strongly influenced by temperature and moisture (Figure 2.1). The eggs are normally 60-80 µm long and thin-shelled. It is not possible to visually identify to genus level at this stage unless lectin egg staining (Palmer and McCombe, 1996) is performed, as the eggs of trichostrongylid species are similar in appearance and overlapping in size. Embryonation (formation of embryo in the eggs) occurs when temperature and moisture are optimum (Veglia, 1916; O'Connor *et al.*, 2006). The embryonated eggs are able to hatch to first stage larvae (L1; 300-400 µm length) in 24 h when temperature, moisture and oxygen are sufficient (Veglia, 1916). The L1 then develop and moult to second stage larvae (L2; 500 µm length) shedding their protective cuticle in the process. The L2 repeat the process to develop to infective L3 (*H. contortus*: 650-751 µm; *T. colubriformis*: 622-796 µm length) in five days under optimum conditions, but development may be delayed for weeks for *H. contortus* and months for *T. colubriformis* in a cold environment (Urquhart *et al.*, 1996). During the moulting process from L2 to L3, the larva retains the cuticle from the previous moult, thus the L3 are more resistant to moisture stress, but temperature may adversely affect the L3 (Hansen and Perry, 1990). The L1-L2 feed on bacteria and other microorganisms in the faeces (Levine and Todd, 1995), and they are very sensitive to environmental stress as during these stages they only have one cuticle. The L3 cannot feed as the mouth is closed (Veglia, 1916) and depend on reserved energy, thus their length of survival depends on how fast they deplete their reserved energy. The higher the temperature the faster the L3 metabolism and the quicker the depletion of energy reserves, leading to death. Development from egg to L3 occurs in faecal pellets and the

time required for development to L3 depends on temperature, such that under optimal conditions (high humidity and warm temperature), the developmental process requires about 4 to 10 days. In contrast, the process may be prolonged in areas with cooler temperatures. A moisture medium such as rain or dew is required for the L3 to migrate out of the faecal pellets.

In Sungai Siput, Malaysia, development of 26% *H. contortus* and 66% *T. colubriformis* eggs to L3 was completed within 9 days but 90% of the L3 had died by the fifth week after faecal deposition (Cheah and Rajamanickam, 1997). Comparatively, development to L3 in colder conditions takes longer to complete but survival on pasture is longer than observed in tropical areas. For example, following deposition during summer, development of *H. contortus* to L3 reached a maximum level after 2-4 weeks and large numbers of L3 continued to be recovered throughout the winter (Rose, 1963).

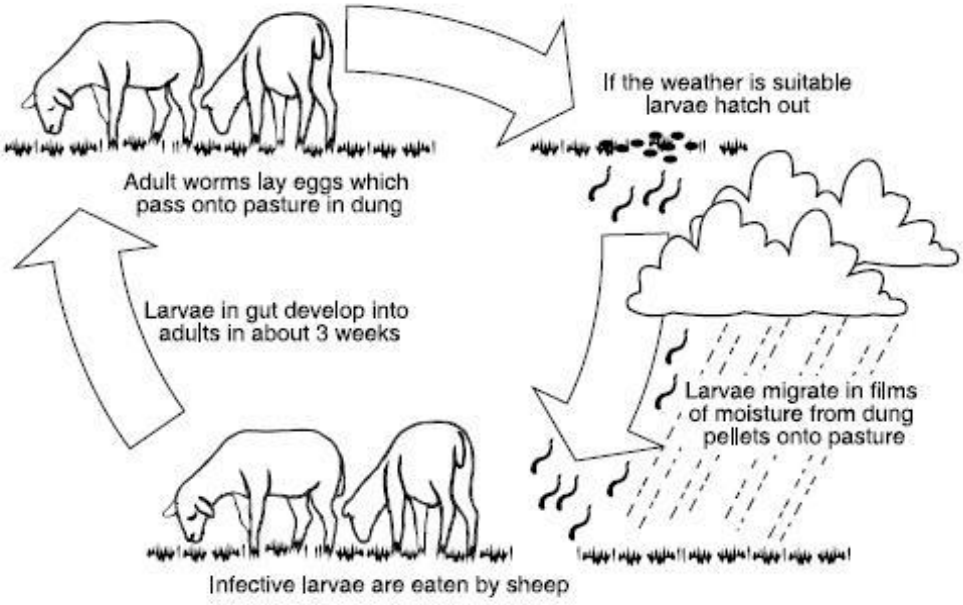

Figure 2.1: The life cycle of parasitic gastrointestinal nematodes is influenced by weather and moisture (Department of Agriculture and Food Western Australia, 2011).

Most L3 are picked up during grazing and eventually passed to the abomasum (*H. contortus*), or small intestine (*T. colubriformis*), ex-sheathing the extra cuticle in the process. The L3 of both *H. contortus* and *T. colubriformis* penetrate the mucous membrane of the gut, and 4-11 days after ingestion by the animal host, L4 emerge (Urquhart *et al.*, 1996). These L4 later establish in the lumen of the abomasum (*H. contortus*) and small intestine (*T. colubriformis*). The L4 of *H. contortus* develop a piercing lancet before the final moulting to the fifth stage larvae (L5) which enables them to ingest blood from the mucosal vessels. Host-animals lose blood from ingestion and also from considerable bleeding due to piercing of the intestinal wall during the ingestion process. One female adult *H. contortus* is able to produce an average of 4,700 eggs daily (Coyne and Smith, 1992a) and up to 10,000 eggs daily for several months in succession (Gordon, 1948). In contrast, a female adult *T. colubriformis* has lower fecundity and produces up to 700 eggs daily (Barnes and Dobson, 1990a).

The period between the infection of an animal by ingestion of L3 and the first egg production by the adult female parasite is called the pre-patent period. The pre-patent period for *H. contortus*, *T. colubriformis* and other GIN in the Family Trichostrongylidea is 16-20 days in sheep (Brightling, 1994; Urquhart *et al.*, 1996). Full egg production will occur approximately on day 25-35 after larval ingestion (Gordon, 1948).

2.4 Effect of infection on production

Infection by *H. contortus* and *T. colubriformis* affects production and in severe cases can lead to mortality. This section is not central to the literature review and only a brief summary is provided.

2.4.1 *H. contortus*

The main clinical feature of *H. contortus* infection is anaemia as the L4 and adult stages ingest blood and leave wounds which cause haemorrhaging into the abomasum. Each adult female nematode is able to remove approximately 0.05 ml of blood per day from its host through a combination of ingestion and leakage from the lesions (Urquhart *et al.*, 1996). Besides anaemia, heavy *H. contortus* infections normally result in haemorrhagic gastritis which leads to death (Urquhart *et al.*, 1996). Heavy infection of *H. contortus* leads to hypoalbuminaemia, a medical condition where the level of albumin in blood serum is abnormally low. Facial oedema (bottle jaw) may develop in severe cases of hypoalbuminaemia (Holmes, 1985). Infection by *H. contortus* can reduce milk production (Sykes, 1983; Holmes, 1985; Parkins and Holmes, 1989) and it has been observed that infection by L3 for a period of 6 weeks prior to and 6 weeks after lambing reduces milk yield by 17 – 23% (Thomas and Ali, 1983; Macarthur, 2010).

Infection by *H. contortus* reduces wool growth. For example, Albers *et al.* (1989) reported following a five week infection of 10,000 *H. contortus* (intraruminal bolus) that clean wool growth was reduced by 6.8% range = 1.4–15.7%) up till four months post infection. The most notable effect of *H. contortus* on production is via an increase in mortality (Kelly *et al*., 2010).

2.4.2 *T. colubriformis*

T. colubriformis infection leads to increased plasma loss and this coincides with the onset of inappetance, hypoproteinaemia and weight loss (Barker, 1973). Heavy infection by *T. colubriformis* is often associated with diarrhoea (Holmes, 1985; Urquhart *et al.*, 1996), though hypersensitivity to low rates of infection of *T. colubriformis* can also lead to diarrhoea (Larsen *et al.*, 1999). Attachment of the adult stage to the small intestine causes severe enteritis (Holmes, 1985) which displaces absorption to more distal regions of the

small intestine (Coop and Angus, 1975) and limits phosphorus absorption (Barker, 1973), which over a prolonged period may result in stunted skeletal growth (Poppi *et al.*, 1985).

Mixed infection with other GIN species often leads to greater negative effect on production and this is most obvious with feed intake. For example Knox and Steel (1999) observed that mixed infection of *H. contortus* and *T. colubriformis* led to a greater reduction in feed intake when compared to infection with *T. colubriformis* alone.

The effect of reduced feed intake accounts for much of the production loss due to *T. colubriformis* infection. Steel *et al.* (1980; 1982) reported that infection with 3,000 – 38,000 L3/week of *T. colubriformis* led to 13–66% reduced wool growth and 20–76% reduced weight gain.

2.5 Environmental factors that regulate developmental rate of *H. contortus* and *T. colubriformis* from egg to L3

Development of GIN from egg to L3 is regulated by environmental factors, mainly temperature and moisture. Rainfall, evaporation rate, the structure of the herbage sward, soil moisture and temperature influence the microclimate (temperature and moisture availability) of GIN eggs in the faecal pellets. The availability of moisture in the faecal pellets can be measured through faecal moisture (FM) which can be influenced by many factors (Figure 2.2).

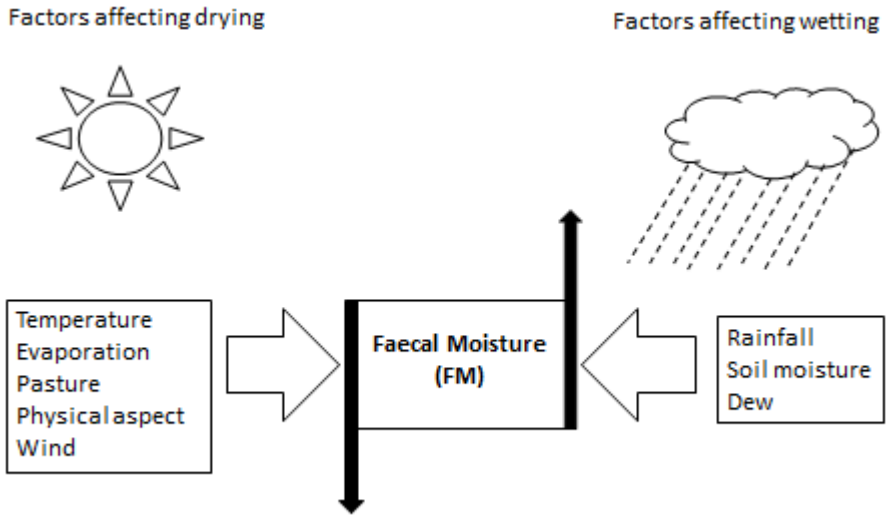

Figure 2.2: Factors affecting the drying and the wetting of faecal pellets. The factors are interrelated; for example rainfall regulates soil moisture and wind regulates evaporation rate. Physical aspect refers to the horizontal direction to which the land faces.

An understanding of the climatic requirements for egg hatching and its development to L3, and survival of both eggs and larvae on pasture is important for successful GIN management. This information enables prediction of the development of GIN on pasture, based on environmental variables such as temperature and moisture, and will assist decision making on drenching and rotational grazing.

Information on the effect of environmental factors, especially temperature and moisture, on development of GIN eggs to L3 on pasture has been incorporated into several prediction models for GIN parasites (Barger *et al.*, 1974; Callinan *et al.*, 1982; Barnes *et al*., 1988; Learmount *et al*., 2006), and most of these models have been validated to some extent. Information on temperature and moisture requirements for development of *H. contortus* and *T. colubriformis* to L3 is readily available in the literature but many of the reports only describe moisture requirements in general and incomplete terms, and as such, are not easily applied to other environments. Lack of specific information (such as

moisture source, rainfall distribution and evaporation rate) leads to errors in the prediction of development success in the field (Levine, 1980). For these reasons the focus in this section is on the role of moisture in development of GIN to L3.

2.5.1 Temperature

Temperature requirements for development of *H. contortus* and *T. colubriformis* from unembryonated eggs to L3 have been reported by many authors for a number of years (Veglia, 1916; Dinaburg, 1944; Swan, 1970 and Beveridge *et al*., 1989) and were recently reviewed by O'Connor *et al*. (2006) (Figure 2.3).

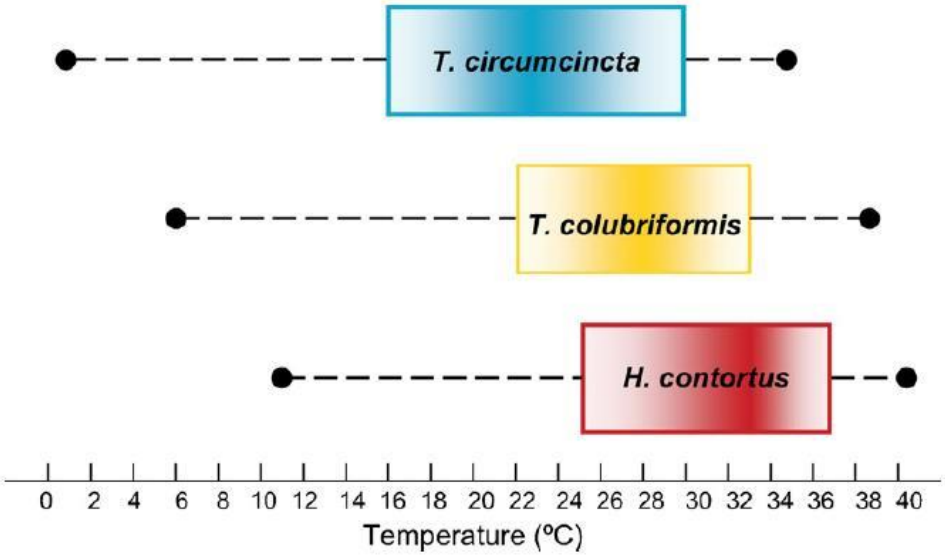

Figure 2.3: Temperature range for development of major trichostrongylid species from unembryonated eggs to L3, with most optimum temperature marked by high colour intensity. Dashed lines (–) extend to the upper and lower temperature limits for development (O'Connor *et al*., 2006).

Generally, *H. contortus* requires a higher temperature range for optimum development and in the range of 95-144 degree days (temperature x number of days). *T. colubriformis* requires fewer degree days (90-115 degree days) to reach the L3 stage under constant temperature or when temperatures are cycled in the range 20-35 °C with 100% relative humidity (Hsu and Levine, 1977). Unfortunately, the types of temperature measurements that have been reported in earlier observations have varied. Temperature measurements for constant temperatures are uncomplicated but daily temperature variation has seen minimum, maximum or mean reported. It has been the mean minimum (Swan, 1970) and mean maximum (Dinaburg, 1944) temperatures that have been used to predict development of *H. contortus*; and both values were incorporated in a prediction model by Barger *et al*., (1974).

2.5.1.1 Constant temperatures

Earlier observations on temperature requirements for *H. contortus* embryonation and hatching were made using constant temperatures. For example, Jasmer *et al*. (1986) reported that *H. contortus* eggs took 96 h to embryonate when incubated at 10 °C. Veglia (1916) observed that freshly deposited *H. contortus* eggs reached the advanced morula stage after 4 h exposure to a constant 26 °C, and by 10 h the embryos showed frequent or constant movements while exposure to a constant 27 °C led to 80% of the eggs hatching. In contrast, the development rate of *H. contortus* eggs to L3 when incubated at constant temperatures of 37 (Berberian and Mizelle, 1957) or 40 °C (Jehan and Gupta, 1974) was 10% and 20% respectively. Optimum development of *H. contortus* eggs to L3 occurs at a constant temperature of 33.3 °C with 100% relative humidity where development of eggs to L3 takes 60-65 h (Berberian and Mizelle, 1957).

T. colubriformis eggs are reportedly able to hatch under a constant temperature as low as 4 °C (Beveridge *et al*., 1989; O'Connor *et al*., 2006). Hatching rates increases from 3.9 to

51.4% and then to 79.2% as constant temperature increases from 4 to 10 °C and finally to 20 °C (Beveridge *et al*., 1989). The maximum temperatures at which *T. colubriformis* eggs hatch is 40.0-44.9 °C (Levine and Andersen, 1973).

2.5.1.2 Other type of temperature measurements

Few authors reported their observation using minimum daily temperature. Donald (1968 – 1969) observed that *H. contortus* eggs have a life span of 5 days and within this period the eggs should be exposed to a minimum daily temperature of 10 °C in order to hatch. However, it has been reported by O'Connor *et al*. (2007a) that some hatching and development to L1 and L2 stages can still occur between day 7 and 14 after faecal deposition (recovery of approximately 3% on day 14) when the minimum daily ground temperature was below 5 °C. A minimum daily temperature of 10 °C is needed for *H. contortus* L1 and L2 stages to develop to L3 (O'Connor *et al*., 2006). Dinaburg (1944) suggested that maximum daily temperature should be 18 °C or greater in order for *H. contortus* eggs to successfully develop to L3.

Embryonation of *T. colubriformis* eggs takes less than 1 day to complete when the mean weekly maximum temperature is 36.8 °C (Levine and Andersen, 1973) or when the mean daily temperature is within the range 13.5-16.6 °C and the maximum temperature equals or exceeds 18 °C (Andersen *et al*., 1966).

Levine and Andersen (1973) observed the developmental speed of *T. colubriformis* L1 and L2 to L3 decreased when the weekly mean maximum temperature increased; it took 63-112 days when the temperature was 4.8 °C and it took 2-4 days to develop to L3 when the temperature was 31.1 °C (Levine and Andersen, 1973). It was reported that weekly maximum temperature must be at least 10 °C to allow *T. colubriformis* embryonated eggs to develop to L3 (Levine and Andersen, 1973).

The different temperature measurements used in the literatures to measure the effects of temperatures on development of eggs to L3 make direct specific comparisons between studies difficult. While constant temperature reported for development of GIN gave us information on the requirements for development to occur, it did not portray they real condition where the GIN eggs were deposited, as in many areas of the world, temperatures fluctuates throughout the day. Thus, mean daily temperatures should be used as a guideline, together with mean minimum and mean maximum, as oppose to constant temperatures. Ground temperatures should be used instead of air or screen temperatures (which were normally measured above 1 m from ground) because it represents the temperature where faecal pellets are deposited (Levine, 1978).

2.5.2 Rainfall

Rainfall – being the main regulator of moisture in the free-living environment – is a key determinant of the successful development of GIN eggs to L3. Rainfall amount, timing and distribution are the critical factors which influence this process. As the free-living stages of *H. contortus* are more susceptible to limited moisture than those of *T. colubriformis*, there are more reports on the effect of moisture on the development of *H. contortus* to L3.

It has been proposed that mean monthly rainfall of 50 mm is needed for optimum pasture transmission of *H. contortus* (Gordon, 1948) and *T. colubriformis* (Levine and Todd, 1975), and this value is widely used as a guideline for the occurrence of infection from trichostrongylids as a whole. The rainfall amount suggested by these authors is a useful guide for prediction of GIN infection, however the developmental response to the suggested amounts depends on other factors such as rainfall days, rainfall timing, rainfall distribution, evaporation rates, soil moisture and herbage height.

Increasing simulated rainfall amount from 12 – 32 mm influenced development of *H. contortus* eggs to L3 but the benefit was greatest under conditions of low evaporation

(O'Connor *et al*., 2007b). In Ghana, the number of *H. contortus* and *T. colubriformis* L3 recovered from pasture displayed a strong positive association with amount of rainfall but was also influenced by the number of rain days (Agyei, 1997). The author showed that a minimal amount of rainfall (*approx.* 25 mm) would be needed before the number of rain days influences availability of L3 on pasture.

Rainfall timing is one of the important factors determining the successful development of *H. contortus* to L3 with moisture shortly after faecal deposition identified as the most important factor for successful *H. contortus* egg development to L1-L2 and L3 (Veglia, 1916; Besier and Dunsmore, 1993; O'Connor *et al*., 2007a). For example, rainfall (6, 12, 18, 24 mm) which occurs soon (days 1-4) after egg deposition will lead to greater development of *H. contortus* eggs than the same amounts applied at 8 or 15 days post deposition. These results suggest that rainfall events occurring more than 1 week after faecal deposition have no (or little) detectable effect on *H. contortus* recovery (O'Connor *et al*., 2007a). The importance of moisture during the first four days after faecal deposition was first suggested by Veglia (1916) when sustained moisture after faecal deposition led to the first L3 being found on the grass during the fourth day. Similarly, in the classic ecological studies of Besier and Dunsmore (1993) where faeces containing *H. contortus* eggs was deposited at monthly intervals for 3 years, the recovery of *H. contortus* L3 from green pasture was highest when rainfall occurred within 3 days after deposition and during warm weather (mean daily temperature of 15.8-20.2 $^{\circ}$C).

In addition to rainfall amount and timing, O'Connor *et al*. (2007a) showed that rainfall distribution may play an important role in successful development of *H. contortus* to L3. When 12, 24 and 32 mm of simulated rainfall were distributed evenly over 3 consecutive days, more L1-L2 were recovered by day 4 compared to a single applications of the same amounts (O'Connor *et al*., 2007a). However, the benefit of rainfall distribution on successful early development of *H. contortus* does not coincide with the subsequent findings of O'Connor *et al*. (2007b) on development through to L3. These authors found

that 12-32 mm rainfall applied as a single event led to greater extra-pellet *H. contortus* L3 in the soil, compared to when rainfall was applied as split events distributed equally over 3 near alternate days (days 1, 3, 6 post deposition). These contradictory findings reported by the same authors could be explained by the different simulated rainfall distributions between the two experiments. In the second study (O'Connor *et al*., 2007b) the split rainfall application on alternate days over 1 or 2 weeks did not provide sufficient moisture in the days immediately after faecal deposition. In contrast, the split rainfall application in the first study (O'Connor *et al*., 2007a) was applied on 3 consecutive days after faecal deposition, allowing sufficient moisture in the crucial first few days following deposition. An earlier study by Besier and Dunsmore (1993) also suggested that rainfall distribution was an important factor for development of the free-living stages of *H. contortus*. When 8 mm of rainfall fell on 4 consecutive days following faecal deposition on pasture plots, recovery of L3 was higher compared to recovery when 26 mm rain fell on the day after faecal deposition.

Development of *T. colubriformis* to L3 on pasture is generally considered to be less influenced by moisture than *H. contortus*. In support of this, no correlation between rainfall and L3 prevalence in the southwest of Western Australia was reported by De Chaneet and Dunsmore (1988). Development of this GIN to L3 is still possible even with low amounts of moisture, as L3 were recovered in Fiji when the monthly rainfall during deposition was less than 10 mm (Banks *et al*., 1990). However, in an earlier study in Urbana, Illinois, it was suggested that a total monthly rainfall of 25 mm was required for development of *T. colubriformis* to L3 (Levine and Andersen, 1973).

The rainfall factors of amount, timing and distribution mediate their effect of development through affecting faecal moisture which is the subject of the next section.

2.5.3 Soil moisture

Aside from rainfall and dew, soil moisture is the only other source of additional moisture for faecal pellets. The humidity in the microenvironment of faecal pellets depends on factors that influence soil moisture (Armour, 1980). Surface soil moisture is influenced by rainfall and evaporation rate (Levine, 1978). On the soil surface, the free-living stages of GIN are more vulnerable to drying conditions compared to those stages in the soil, where larvae are protected from desiccation as the relative humidity in the soil even at the wilting point is over 98% (Wallace, 1961). Moisture in the soil also provides moisture indirectly to the GIN eggs in the faecal pellets by capillary action, reduces the ground surface temperature and provides moisture to the pasture that will protect the faecal pellets from drying (Bullick and Andersen, 1978).

Veglia (1916) reported that on the third day after deposition, “high numbers” of L2 were found in the faeces at the lower part of the pellets which were in contact with the soil, compared to the upper part of faecal pellets. By the fourth day, 80% of the *H. contortus* larvae were found in the lower layer of the faecal pellets. This might be explained by the fact that the lower part of the faecal pellets in contact with the soil had sufficient moisture and were more protected from drying than those not parts not touching the soil surface. In contrast, soil moisture in Urbana, Illinois, was not an important factor in successful development of *T. colubriformis* to L3 (Levine and Andersen, 1973) probably because soil moisture never went below 12% and was always sufficient for development to L3 to occur. In a prediction model for development of *Teladorsagia* (*Ostertagia*) and *Trichostrongylus* spp., soil moisture was considered one of the most important factors along with air temperature for development to L3 (Callinan *et al*., 1982). This is in agreement with Levine and Todd (1975) who suggested that temperature and soil moisture are the most important factors affecting development and survival of *H. contortus*. The authors stated that soil moisture is more important than rainfall alone for the development and survival of *H. contortus,* as soil moisture is influenced by the interaction of rainfall, soil type and

evapotranspiration. Soil moisture also reflects a moisture regime over a longer period which suggests that it is a good predictor for moisture availability, subsequently development to L3, but detailed information on this is yet to be reported.

Given the importance of soil moisture, rainfall which occurs prior to faecal deposition may also provide benefit for the development of GIN through increased soil moisture and changes in herbage but such putative effects remain without support and are yet to be tested experimentally.

2.5.4 Evaporation

Evaporation has been considered as an important factor in prediction models of L3 on pasture (Barger *et al*., 1974; Barnes *et al*., 1988) (Sections 2.7.1 and 2.7.3).

Evaporation involves vaporisation at the surface of a liquid. It is influenced by many factors, but mainly temperature and wind. Evaporation rates can regulate the effect of rainfall amount on development of eggs to L3. Interactions between rainfall and evaporation will limit the development of *H. contortus* to L3 (Krecek *et al*., 1992; Besier and Dunsmore, 1993). For example, 32 mm of simulated rainfall under conditions of high evaporation (4.8 mm/day) was observed by O' Connor *et al*. (2008) to be equivalent to 24 mm under low evaporation (2.5 mm/day) for development of *H. contortus* to L3. Unlike *H. contortus*, embryonated eggs of *T. colubriformis* are able to tolerate greater evaporation at temperatures 20-30 °C (Waller and Donald, 1972) which is consistent with the understanding that the development of this GIN species is more tolerant of desiccation.

2.5.5 Herbage mass and herbage height

Herbage influences GIN development by modifying the temperature and moisture (humidity) in the environment of the eggs (Crofton, 1949; Armour, 1980; Sakwa *et al*.,

2003b). A higher mass of herbage (Aumont and Gruner, 1989) and a higher proportion of green leaf (Besier and Dunsmore, 1993) will protect the faecal pellets from drying rapidly and provide sufficient moisture in the absence of rainfall. In contrast, short vegetation exposes faeces and free-living stages to sunlight and desiccation (Gordon, 1948). Herbage microclimate as represented by a range of herbage heights (3, 9, 15 cm) was suggested to affect *H. contortus* egg hatch success as more L1 were recovered from faecal pellets deposited on 9 and 15 cm swards compared to 3 cm swards (Sakwa *et al*., 2003a). The effect of herbage height was likely mediated through the direct effect of temperature and its associated effect on the rate of drying of soil and faecal moisture. For example, ground temperatures (during the seasons of autumn, winter and spring) reported in the 15 cm swards were up to 10 °C higher for daily minimum and lower for daily maximum values that were reported for the 3 cm sward (Sakwa *et al*., 2003b). Earlier, Dinaburg (1944) suggested that *H. contortus* developed better to L3 when faecal deposition occurred under shade (0.003% development compared to development of 0.001% when deposition occurred in unshaded areas) presumably because of lower daily maximum values and effects on prolonging FM.

In addition to the height of the herbage, increasing the amount of green herbage will increase the development success of L1-L2 (O'Connor *et al*., 2007a) and L3 (Besier and Dunsmore, 1993) stages on pasture. Barger *et al*. (1974) made an assumption, in the absence of data on humidity within the herbage from their own study, that there is a positive relationship between humidity and green herbage mass. Mean pasture moisture scores were found to be highly correlated with *H. contortus* L3 recovery from herbage (Besier and Dunsmore, 1993), suggesting that this measurement could be used to predict successful development of *H. contortus* to L3. A high density of herbage combined with rainfall will also lead to greater development of *H. contortus* to L3 (Gruner *et al*., 1989).

2.5.6 Faecal moisture

As GIN eggs are passed from the host's gut in the faeces, FM is the main indicator of the moisture availability in the microenvironment of the eggs.

FM of freshly deposited sheep faecal pellets has been reported as 51-65% (Gruner and Suryahadi, 1993), 53% (O'Connor *et al*., 2007b), 54-60% (Chiejina *et al*., 1989), 60% (Berbigier *et al*., 1990) and 70% (Williams *et al*., 2010). Whether sheep have been consuming fresh forage or dry hay has a large effect on FM and possibly accounts for some of this variation in FM.

The minimum FM which permits 1% of *H. contortus* eggs to develop to L3 has been identified as 39% while FM of 35% was needed for *T. colubriformis* (Rossanigo and Gruner, 1995). FM of 70% when the temperature was 23 °C is optimal for the development of *H. contortus* to L3, while for *T. colubriformis* FM of 55-60% is optimal at a temperature of 28 °C (Rossanigo and Gruner, 1995). Berbigier *et al*. (1990) found that only 6% of *H. contortus* and 36% *T. colubriformis* eggs remained viable 48 hours after deposition at 21 °C with faecal moisture falling below 5%.

O'Connor *et al*. (2007a; 2008) suggested that the initial moisture in normal faecal pellets was sufficient for development of at least 0.19% of *H. contortus* eggs to L1 and L2. However, without additional moisture few (0.55%) L3 developed and no L3 were able to emerge from the drying faecal pellets when recovered at day 14 after faecal deposition (O'Connor *et al*., 2008). A possible explanation for trapped L3 in faecal pellets is the presence of a hardened surface due to desiccation, as van Dijk and Morgan (2011) observed that only 0.18% of *H. contortus* L3 successfully emerged from desiccated faecal pellets to be recovered from grass compared to wet faecal pellets (3.43%). Further support for the requirement for FM to be sustained through additional moisture sources is evinced by a positive association between FM on day 4 and *H. contortus* L3 recovery from the soil (O'Connor *et al*., 2007b).

2.5.7 Cumulative precipitation-evaporation ratio (P/E)

Precipitation (i.e. rainfall; P) and evaporation (E) interact to regulate the moisture environment and L3 development when temperature is not limiting. Higher amounts of P and lower rates of E will lead to greater development of *H. contortus* and *T. colubriformis*, as discussed in section 2.5.2 and 2.5.5. Barger *et al*. (1974) suggested that the cumulative ratio of P/E would be a useful predictor for development of *H. contortus* to L3 on pasture by integrating the opposing effects of P and E. The authors argued that the time taken for P/E to exceed 1 will determine the percentage of successful development of *H. contortus* to L3. They suggested that, in an environment with mean maximum temperature was equal to or greater than 18 $^{\circ}$C, if P/E exceeded 1 after one week post faecal deposition, 90% of the eggs would develop to L3. If it took two weeks to exceed a value of 1, only 70% would develop to L3, and 50% would develop to L3 if it took 3 weeks to exceed the P/E value of 1.

Recent findings by O'Connor *et al*. (2008) reported that even when P/E ratios were strongly positive, less than 40% of the pre-infective stage developed to L3, indicating that the prediction model (Barger *et al*., 1974) overestimated the developmental success to L3. Despite overestimating development success, the cumulative P/E ratio at day 3 post deposition was linearly associated with L3 development and was a significant (R^2=0.45) predictor of development success. It is possible that the predictive model of Barger *et al*., (1974) did not sufficiently account for the importance of P occurring within a few days of deposition on development to L3. An emphatic demonstration of the importance of early rainfall on *H. contortus* L3 was provided by O'Connor *et al*., (2007a) as a repeat application of rainfall (12, 24 or 32 mm) in the second week after deposition gave little benefit for L3 recovery.

In a different approach to using the ratio of P/E to predict development from that originally proposed, O'Connor *et al*. (2008) calculated the importance of the time taken for the

cumulative ratio of P/E to fall to a value below 1. When this occurred, development of *H. contortus* L3 occurred in only 30% of cases and never exceeded 0.1% (Figure 2.4).

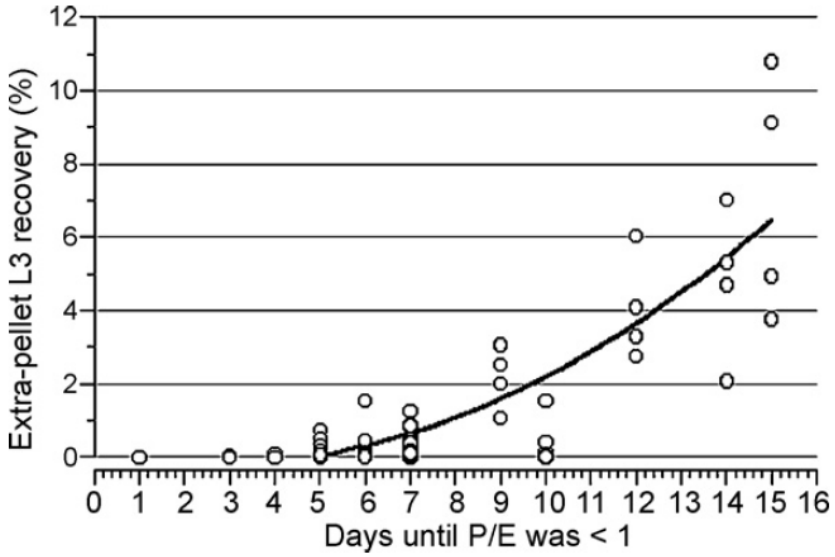

Figure 2.4: Regression of recovery of extra-pellet L3 (averaged over time; arithmetic means) with days until cumulative P/E was less than 1. Polynomial fit to data indicated by solid line ($L3\% = 0.042P/E^2 – 0.21P/E$; $R^2 = 0.77$; $P < 0.001$) (O'Connor *et al*., 2008).

Aware of the difficulties poised by the use of ratios (e.g. uninformative of changes in numerator or denominator), Barnes *et al*. (1988) used the difference between rainfall and evaporation after faecal deposition in the *T. colubriformis* prediction model. The correlation between p_2; the probability that an egg develops to L3 and migrates to the herbage, and *d*; the cumulative value of evaporation-rainfall for 1-7 days was examined. The authors reported that p_2 was best associated with cumulative value of *d* over the first 2 days after the eggs were deposited on the pasture.

2.6 Environmental factors that regulate the mortality rate of *H. contortus* and *T. colubriformis* developmental stages from egg to L3

2.6.1 Temperature

For *H. contortus*, unembryonated eggs were identified as the most cold-susceptible of the free-living stages, followed by L2, L1, embryonated eggs and finally L3 (Todd *et al*., 1976). Silverman and Campbell (1959) reported that at a constant temperature of 7.5 °C, 90% of *H. contortus* eggs did not embryonate, and presumably died. Mortality of *H. contortus* eggs was observed when incubation occurred at a constant 5.6 °C for 120 h (Berberian and Mizelle, 1957). When eggs were incubated at a constant 5 °C for 22 days, no larvae were recovered and the eggs had degenerated (not viable) at the end of the incubation period (Coyne and Smith, 1992b). Temperatures below 4 °C are lethal to *H. contortus* eggs when exposed for 82 h (Berberian and Mizelle, 1957) while Veglia (1916) and Todd *et al*., (1976) demonstrated 4 °C was lethal to eggs only exposed for a few hours. Coyne and Smith (1992b) reported that temperatures above 36 °C are fatal for the development of *H. contortus* to L3 (Coyne and Smith, 1992b), however Veglia (1916) suggested that a temperature of 42 °C is fatal for the eggs. The latter reported that only a "few" *H. contortus* eggs developed to L3 at 37 °C while eggs failed to develop at 40 °C (Veglia, 1916). Temperatures exceeding 40 °C were lethal for *H. contortus* L3 as Jehan and Gupta (1974) reported 80% mortality of L3 when exposed to a constant 45 °C.

For *T. colubriformis*, unembryonated eggs are more susceptible than embryonated eggs and L1 are more susceptible than L2 at most temperatures (Andersen *et al*., 1966). Embryonated and unembryonated *T. colubriformis* eggs have greater tolerance to cold compared to *H. contortus* eggs, with high mortality only occurring when temperatures fall below 5 °C (O'Connor *et al*., 2006). Exposure to constant 50 °C is fatal within 24 h to all stages of *T. colubriformis* (Andersen *et al*., 1966). Most of the reports have described the

direct effect of temperature on the development of GIN eggs to L3, without considering moisture as a secondary factor as temperatures regulate drying.

2.6.2 Moisture

Generally for *H. contortus* and *T. colubriformis*, the pre-infective stages are the most susceptible stage to desiccation, followed by unembryonated egg, embryonated egg and L3 (Andersen and Levine, 1968; O'Connor *et al*., 2006). Andersen and Levine (1968) observed that all *T. colubriformis* unembryonated eggs were dead after 48 hours when relative humidity was 65-75% and temperature held at 30 °C. Similar results were observed by Waller and Donald (1970) when they observed mortality of all *T. colubriformis* unembryonated eggs after 48 hours exposure to 56% relative humidity at 20 °C. In the absence of rain for 48 h in a tropical climate only 94% of *H. contortus* eggs died (Berbigier *et al*., 1990).

Although moisture is needed for successful development to L3, too much moisture will cause mortality of *H. contortus* eggs as the eggs are susceptible to immersion in water (Gruner and Suryahadi, 1993). When faeces were immersed in water for 7 and 16 h then dried to 60% FM, fewer *H. contortus* L3 were recovered compared to samples not immersed in water and maintained at 60% FM (Gruner and Suryahadi, 1993). This finding is in agreement with Veglia (1916) who reported that when faecal pellets were soaked in water deeper than 50 mm, only a small proportion of *H. contortus* eggs survived and hatched, and within two weeks the eggs died due to lack of oxygen.

2.6.3 Faecal moisture (FM)

FM is an alternative to relative humidity way to describe the moisture content of the pellet microclimate and is an important factor for mortality of the free-living stages (Berbigier *et*

al., 1990). FM lower than 50% during the first 3 days after deposition led to 55% and 38% mortality rate for *H. contortus* and *T. colubriformis* eggs respectively (Berbigier *et al*., 1990). The number of extra-pellet *H. contortus* L3 developed by days 7 and 14 after faecal deposition was nil or very low (less than 1%) when the FM value on day 4 dropped to below 10% (O'Connor *et al*., 2008). Similarly, during the dry season in Nigeria where goat faeces with an initial moisture content of 54-60% lost all moisture within 3 days of deposition, there was no recovery of *H. contortus* and *T. colubriformis* L3 (Chiejina *et al*., 1989).

2.7 Prediction models of free-living parasitic gastrointestinal nematodes on pasture involving moisture

All models that have been developed to predict development and availability of GIN in sheep have included moisture as an input factor. These models were designed to ultimately assist farmers with GIN control through decisions with grazing and drenching management. A brief review of four prediction models was included in this thesis as example of how moisture was accounted for predicting L3 development (Table 2.1).

Table 2.1: Some of prediction models for *H. contortus* and *T. colubriformis*

Model	Species	Variables used to predict development	Precedent rainfall	Soil moisture	Validation with grazing animals
Simulation of *H. contortus* L3 population	*H. contortus*	Precipitation Evaporation Temperatures Herbage mass/Humidity	No	No	No*
NEMAT	*Teladorsagia* spp. and *Trichostrongylus* spp.	Temperature Soil moisture Humidity	No	Yes	Yes
Prediction of *T. colubriformis* L3 population on pasture	*T. colubriformis*	Temperature Precipitation Evaporation	No	No	Yes**
Simulation of parasitic gastroenteritis in sheep	*Haemonchus* spp, *Trichostrongylus* spp. and *Teladorsagia circumcincta*	Temperature Rainfall	No	No	No

* Validated with pasture L3 recovery.** Validated with grazing animals in WormWorld (Barnes and Dobson, 1990b)

2.7.1 Simulation of *H. contortus* L3 population

The model developed by Barger *et al*. (1974) uses the cumulative ratio of precipitation and evaporation (P/E) as an important variable to determine the successful development of *H. contortus* eggs deposited in faecal pellets on pasture to the L3 stage. The critical P/E values for development are described in section 2.5.7.

According to the authors, a P/E value of 1 is required for successful development to L3. In this model, time taken for the P/E value to exceed 1 is a predictor of the percentage of *H. contortus* development to L3 on pasture. Besides P/E, the other moisture variable is humidity based on herbage mass. Death rate of the L3 population is predicted based on the maximum daily temperature and relative humidity. This model was validated by comparing predicted values of pasture L3 against actual pasture larval recovery (but not with grazing animals). There was a strong association (R^2=0.86) between predicted and observed values but the number of assumptions (principally for L3 development) raises questions about the robustness of a generalised fit for *H. contortus* development. If the model did overestimate L3 development (as proposed by O'Connor *et al*., 2008) then it is possible that L3 death rates in the model may also be overestimated in order to explain the good fit between predicted and observed L3 on pasture. While cumulative P/E accounts for post depositional events, there was no allowance for pre-depositional events that may influence soil moisture and hence FM and L3 development.

2.7.2 NEMAT

This model was constructed by Callinan *et al*. (1982) to predict development of *Ostertagia* spp. and *Trichostrongylus* spp. on pasture in western Victoria and to optimise control programs. Mean air temperature and soil moisture were used to determine the development and death rate of both genera, while the distribution of L3 on pasture was predicted by mean daily air temperature and relative humidity. Unlike the model of *H.*

contortus development, NEMAT included prediction of pasture growth rate and live weight of weaner sheep. This model was validated in the field where predicted and observed values for worm egg count were observed to be in the range 0.45 – 0.83. However, the generalised prediction for *Trichostrongylus* spp. as opposed to separate estimates (models) for *T. colubriformis* and *T. vitrinus* were seen as required improvements (De Chaneet and Dunsmore, 1988). Surprisingly, rainfall was not included in the model but the inclusion of soil moisture and the lower sensitivity of both *Trichostrongylus* spp. and *Teladorsagia* spp. development to rainfall may account for its useful predictive value.

2.7.3 Prediction of *T. colubriformis* L3 population on pasture

This model was constructed to predict the population of *T. colubriformis* L3 on pasture based on meteorological data. Evaporation and rainfall in the first 2 days after faecal deposition and the time taken until an effective rainfall event (16 mm) were the moisture variables used to predict the probability that an egg successfully develops to L3 and migrates to the herbage.

Availability of moisture in the microclimate of GIN eggs was used to predict successful development to L3, and was calculated based on cumulative evaporation rates minus the cumulative rainfall values for the first 2 days after faecal deposition. Successful migration of L3 was dependent on moisture availability at the pasture level, and was predicted based on the time taken (in weeks) until the total rainfall in 7 consecutive days exceeded 16 mm. The period of 7 days was without empirical basis and randomly chosen but the amount of 16 mm was chosen based on the best fit between the probability of migration on herbage and the total amount of rainfall in 7 days, although rainfall amounts of 10-20 mm gave similar prediction results. Precedent rainfall and soil moisture were not included in the model. Exploration of other weather variables and pasture conditions which affect the moisture content of faeces was suggested by the authors, due to the low precision of

estimation when using this model. The model was validated with grazing animals in WormWorld (Barnes and Dobson, 1990b). As this model only predicts *T. colubriformis* population, further work was suggested to predict population of *H. contortus* and *O. circumcincta*.

2.7.4 Simulation of parasitic gastroenteritis in sheep

This model was constructed by Learmount *et al*. (2006) to predict development of *Haemonchus* spp., *Trichostrongylus* spp. and *Telodorsagia circumcincta* in the United Kingdom, with temperature as the main factor for free-living development to L3 on pasture. Mortality of L3 was assumed to be dependent on rainfall if the value was less than 50 mm per month. Due to the differing effects of environmental variables on different species of the same genera, this model could be improved if prediction of each species was modelled. Precedent rainfall and soil moisture were not included in the model and the model is yet to be validated with grazing animals.

2.8 Benefits from prediction models

Tropical countries located between 23°26′16″N and 23°26′16″S, where the climate is warm to hot and humid year-round, are favourable for successful development of *H. contortus* and *T. colubriformis*. In an epidemiological study in Selangor on Peninsular Malaysia (Dorny *et al*., 1995), individual maximal worm egg counts of at least 10,000 eggs per gram and associated clinical signs were observed in sheep and goats during the wettest months. Prediction based on climate in this environment is more straight-forward as strongyle infection rate tends to remain the same throughout the year because maximum temperature always exceeds 18 °C and mean monthly rainfall almost always exceeds 50 mm (Dorny *et al*., 1995).

However, in temperate countries where daily temperatures fluctuate and vary between seasons and rainfall events are less frequent, development of GIN to L3 is more sporadic. Under these conditions prediction models based on climate will assist in drenching decisions and grazing management allowing more efficient worm control.

2.9 Conclusions

There is considerable information on the temperature and moisture requirements for successful development of *H. contortus* and *T. colubriformis* to L3. The effect of temperature has been more thoroughly reported and is a less complicated variable: though there is a need for reports to provide at least minimum and maximum daily temperatures. In contrast, the effect of moisture has a number of factors which can regulate development of GIN eggs to L3 and these have been discussed in this review. It is apparent that uncertainty remains over a number of moisture factors that may be important for GIN development and these will form the basis of the experiments described in this thesis. The key research questions are;

1. Does rainfall which occurred on days before faecal deposition influence development of *H. contortus* and *T. colubriformis* to L3?
2. Does soil moisture change the sensitivity to rainfall events of *H. contortus* and *T. colubriformis* development to L3?
3. Is FM suitable to integrate moisture effects and allow prediction of the development of *H. contortus* and *T. colubriformis* to L3?
4. Will the moisture factors used to predict development of *H. contortus* and *T. colubriformis* L3 apply when determining translation to grazing animals in the field?

Chapter 3 Materials and methods

3.1 Study site

Experiments in Chapters 4 and 5 were conducted in climate-controlled chambers (SANYO Electric Biomedical Co., Ltd., Japan) (Figure 3.1a), using CALgrafix Standard 1.1.04 software (CALControls Inc., United Kingdom) to simulate temperature. The experiment in Chapter 6 was conducted in a climate-controlled chamber/plant growth cabinet (Thermoline Scientific, Australia) (Figure 3.1b). The field experiment as described in Chapter 7 was conducted on 21 m x 15 m pasture plots at Laureldale Research Station, University of New England, Armidale (Figure 3.1c).

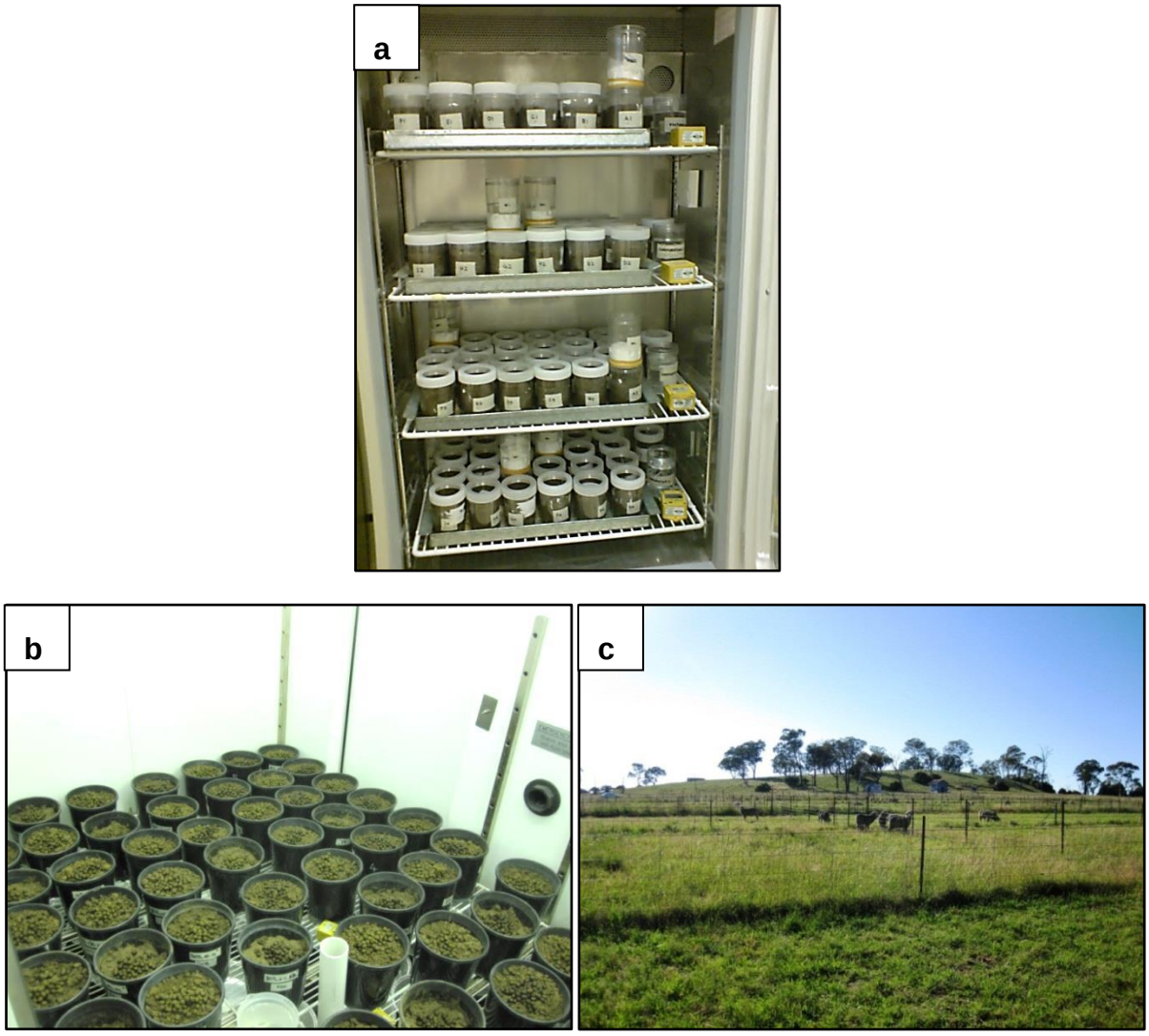

Figure 3.1: Study sites for experiments in Chapters 4, 5 (a), 6 (b) and 7 (c).

3.2 Faecal deposition

Development from egg to L3 occurred in experimental units or on pasture as described respectively in Chapters 4, 5, 6 and 7 (Figure 3.2).

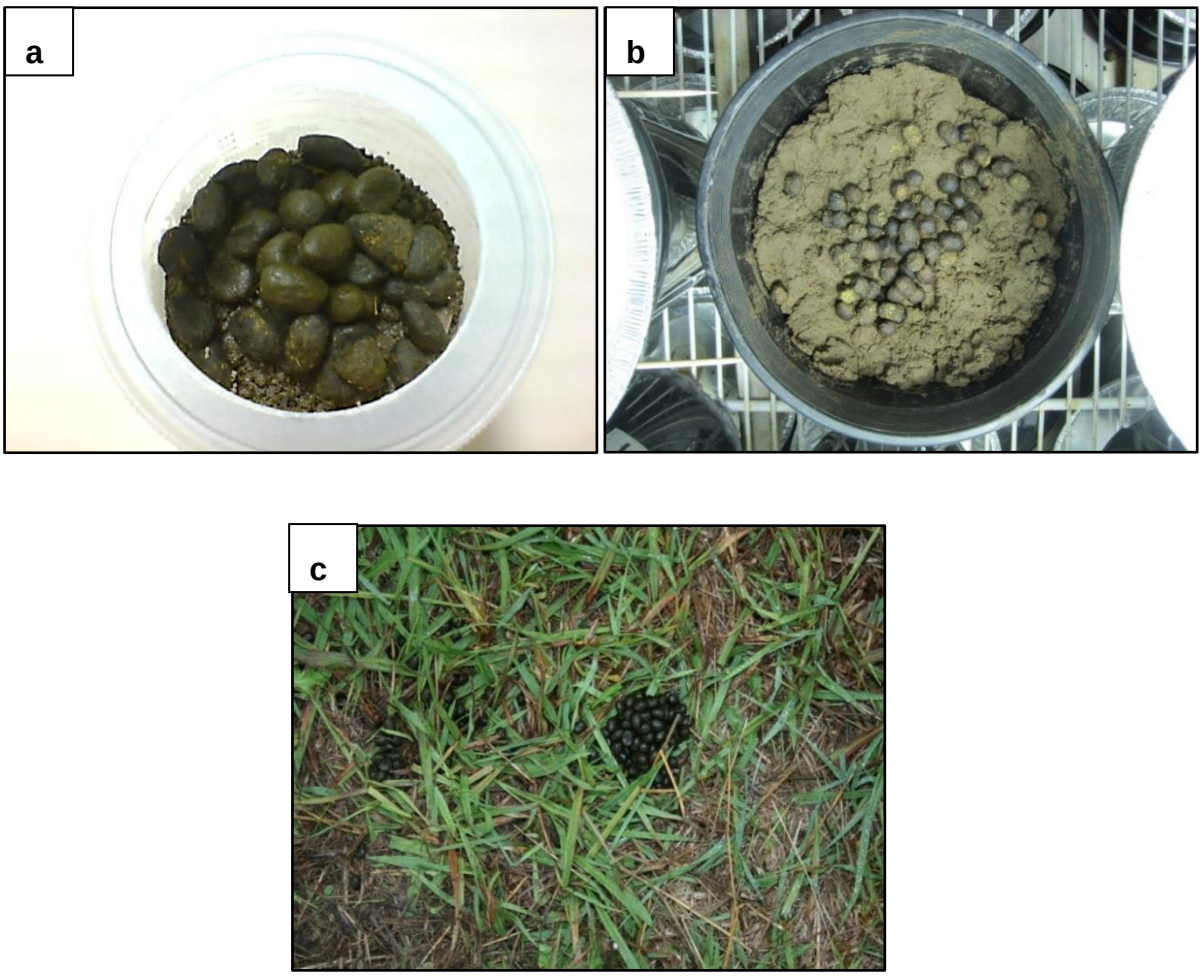

Figure 3.2: Experimental units described in Chapters 4, 5 (a), 6 (b) and 7 (c).

3.3 Rainfall simulation

Simulated rainfall was applied to the experimental units as described in Chapters 4, 5, 6 and to pasture as in Chapter 7 (Figure 3.3).

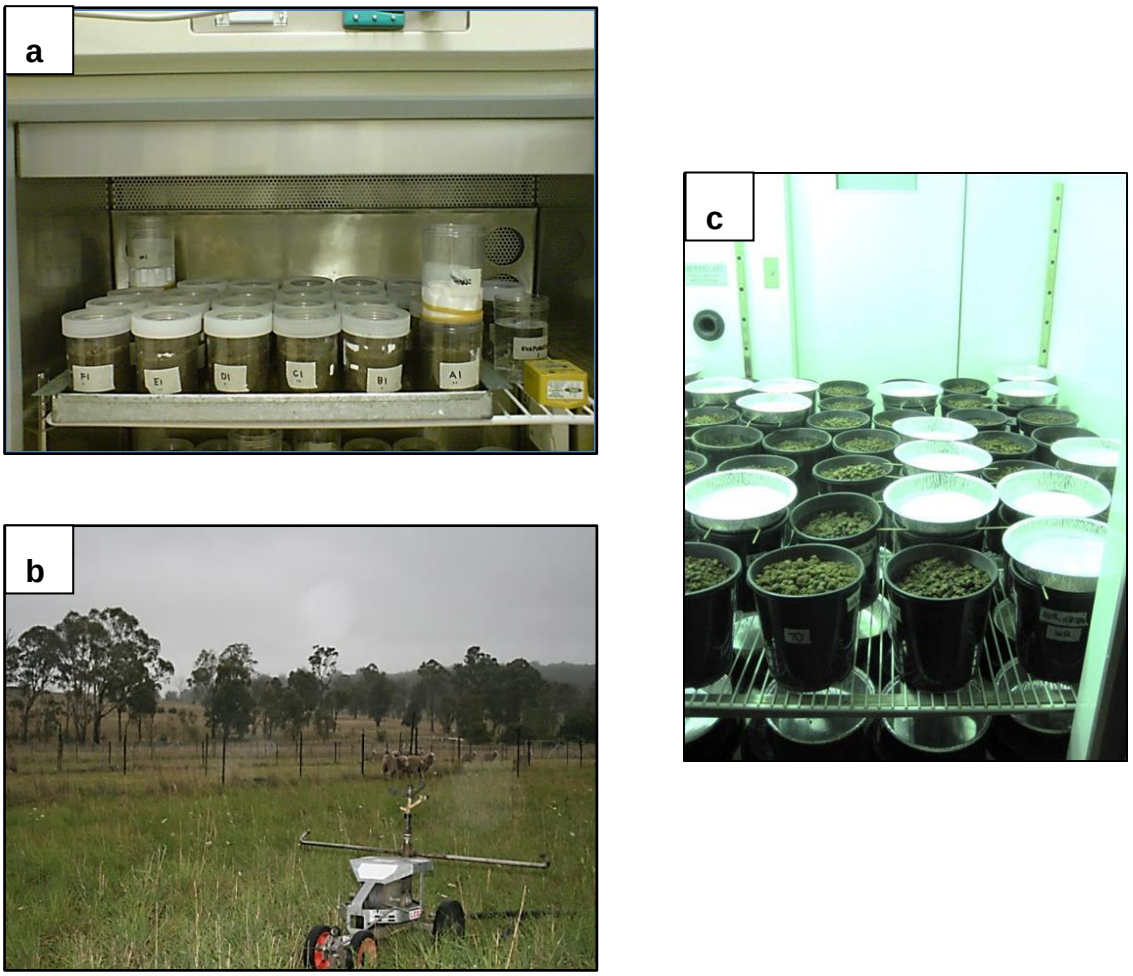

Figure 3.3: Simulated rainfall described in Chapters 4, 5 (a), 6 (b) and 7 (c).

3.4 Faecal moisture

Faecal pellets (n=5) were weighed (A) using a four decimal balance (Mettler Toledo International Inc.) and then dried at 80 °C in an oven (Scientific Equip Pty. Ltd. Australia) for 5 days. Faecal pellets were then reweighed (B) and the moisture content was calculated using the formula:

$$\text{FM}\ (\%) = \left(\frac{\text{A} - \text{B}}{\text{A}}\right) \text{X}\ 100$$

3.5 Soil moisture (Chapter 5)

Soil was weighed (A) using a four decimal balance (Mettler Toledo International Inc.) and then dried at 80 °C in an oven (Scientific Equip Pty. Ltd. Australia) for 5 days. Soil was then reweighed (B) and the moisture was calculated using the formula;

$$\text{Soil moisture}\ (\%) = \left(\frac{\text{A} - \text{B}}{\text{A}}\right) \text{X}\ 100$$

3.6 Herbage mass (Chapter 7)

The median quadrat technique (Department of Primary Industries, 2007) was applied on each plot to estimate herbage mass (kg DM/ha). In each plot, three sampling locations were randomly selected and for each location five subquadrats were sampled (0.25 m^2 each). Herbage with median height in the subquadrats was cut and weighed separately. The samples were then gathered and dried at 70 °C for at least five days to determine the dry matter content from three different locations within each subplots.

Dry matter content (DM) was calculated as;

$$\text{DM}\ (\%) = \left(\frac{\text{Weight of dry sample (g)}}{\text{Weight of wet sample (g)}}\right) \times 100$$

Herbage mass was calculated as;

$$\text{Herbage mass (kgDM/ha)} = \frac{\text{weight of dry sample (kg)}}{0.25} \text{ x } 10{,}000$$

3.7 Parasitological methods

3.7.1 Worm egg count (Chapters 4-7)

Nematode worm egg count (WEC) was conducted on 1.5-2.5 g of faeces following a modified McMaster method (Whitlock, 1948). Distilled water was added to faecal samples in a 5:1 ratio and ground into uniform slurry. The faecal slurry was sieved using a 600 µm mesh and 150 µl of the slurry with 600 µl of saturated salt was transferred to one McMaster counting chamber. Worm eggs were observed and counted under 40x magnification using a compound microscope (Olympus, Japan). The sensitivity of this method is 1 egg equivalent to 60 eggs per gram (epg) of faeces. In Chapter 7, replicates were counted which increased the sensitivity to 1 egg equivalent to 15 epg for tracer animals.

3.7.2 Coproculture and L3 differentiation (Chapters 4-7)

A subsample of faecal pellets was crushed, mixed with vermiculite and sufficient water added to create a moist mixture. The mixture was transferred to 750 ml glass jars and then placed in an incubator (SANYO Electric Biomedical Co. Ltd. Japan) at 25 °C. After 7 days, the samples were removed from the incubator and the jars filled with water. A petri dish was placed over the top and the culture inverted to allow the L3 to sediment. Further water was added to the petri dishes to facilitate larval collection. Each culture was allowed to settle on the bench for a minimum of 1 h before L3 were collected using a Pasteur pipette from the petri dish to a 15 ml centrifuge tube. A drop of larval solution was placed on a glass slide and a drop of Lugol's iodine was added to the solution to kill and stain the

L3. A total of one hundred L3 were identified based on identification keys (Whitlock, 1960). The species composition was recorded in percentage (%).

3.7.3 Total egg output (Chapter 7)

Total egg output for *H contortus* and *T. colubriformis* per plot was calculated using the formula;

WEC × SP × DO,

Where WEC is the worm egg count (epg) of each donor sheep during plot contamination (mean of days -3 and 2), SP is the percentage (%) of *H. contortus* and *T. colubriformis* associated with each WEC as determined by coproculture and species differentiation; DO is estimated total daily output (g) of fresh faeces.

Daily faecal output for donor animals was calculated from that reported by Bailey (2008) after adjustment on the basis of metabolic weight ($W^{0.75}$) and a value of 85 g fresh faeces/$W^{0.75}$/day was used.

3.7.4 Intra-pellet enumeration (Chapters 4-6)

Approximately 1.2 – 3.0 g of faecal pellets were collected and de-ionised water added at a ratio of 21:1. The samples were then ground and filtered through a 600 µm mesh. Sixty (60) µm of the filtrate was dispensed on an etched glass slide with two drops of Lugol's iodine. Degenerate and embryonated eggs, L1-L2 and L3 (Figure 3.4) were identified as described by (O'Connor, 2007) and counted under 40x magnification using a compound microscope (Olympus, Japan).

Recovery was calculated as;

$$\text{Recovery} = \left[\frac{\text{Total number of eggs or larvae counted}}{\text{Total number of eggs deposited}}\right] \times 100$$

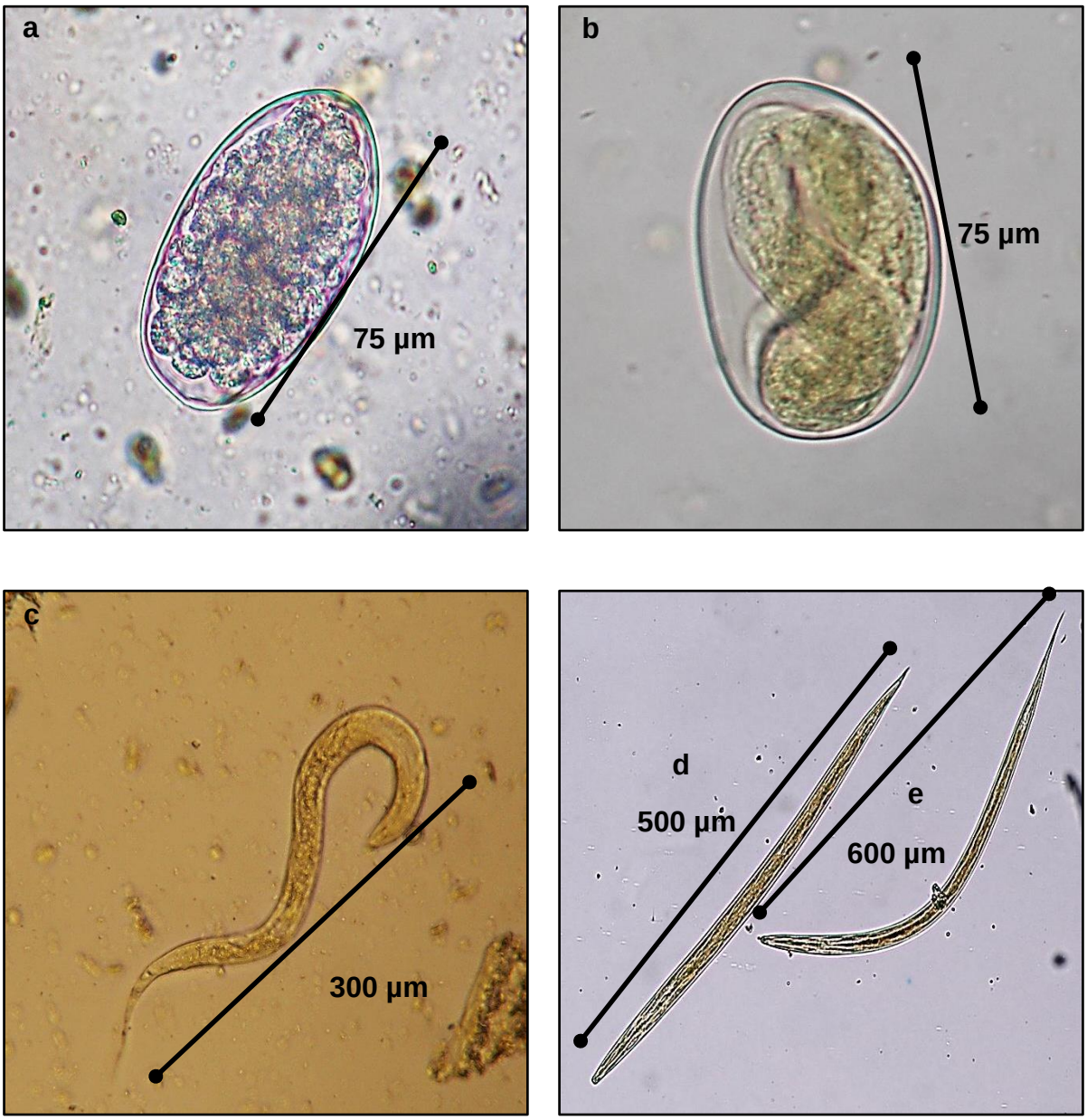

Figure 3.4: Unembryonated egg (a), embryonated egg (b), pre-infective larvae (c) and third stage larvae of *T. colubriformis* (d) and *H. contortus* (e).

3.7.5 Extra-pellet enumeration (Chapters 4-6)

Extra-pellet L3 on day 14 were enumerated using a modification of the method of O'Connor *et al*. (2008), where subsamples were quantified by weight rather than volume. The top 25 mm of the soil was transferred to a 250 ml polycarbonate jar and saturated

with 70% ethanol. The samples were allowed to sediment until processing. The excess alcohol was removed and approximately 10 ml of water was added to create slurry. The slurry was weighed and recorded (A). After mixing by inversion, a 3 ml subsample of the slurry was transferred to a 15 ml tube and 7 ml of potassium iodide (K.I) solution (r.d. 1.4) added for the first float. The samples were then centrifuged at 1400 g for 6 minutes and the supernatant collected. De-ionised water was added to the supernatant up to 50 ml and then centrifuged again for 1400 g for 6 minutes. The supernatant was removed, leaving 0.8-1.5 ml (B) larval sediment and water. The soil sediment from the first float was floated for second time following the same method for the first float to collect any remaining L3. A total of 0.2 ml of the larval solution was transferred to an etched glass slide with two drops of Lugol's iodine. L3 were identified and counted under x 40 magnification. The number of L3 in each treatment plot for each float was calculated as:

$$\text{Larvae per experimental unit} = \left[\frac{\text{A}}{\text{3ml subsample (g)}}\right] \times \left[\frac{\text{B}}{\text{Volume examined (0.2ml)}}\right] \times \text{L3 per chamber}$$

Validation of the above method produced an average percentage of recovery 83% (coefficient of variation = 10%) (Appendix 1).

3.7.6 Total worm count and worm identification (Chapter 7)

Gut contents were collected following the method of Wood *et al*., (1995). *H. contortus* and *T. colubriformis* were identified, enumerated, and differentiated either fourth stage larvae (L4), immature adult, male adult or female adult (Figures 3.5 and 3.6) (MAFF, 1986) using a stereo microscope (Wild Leitz, Australia).

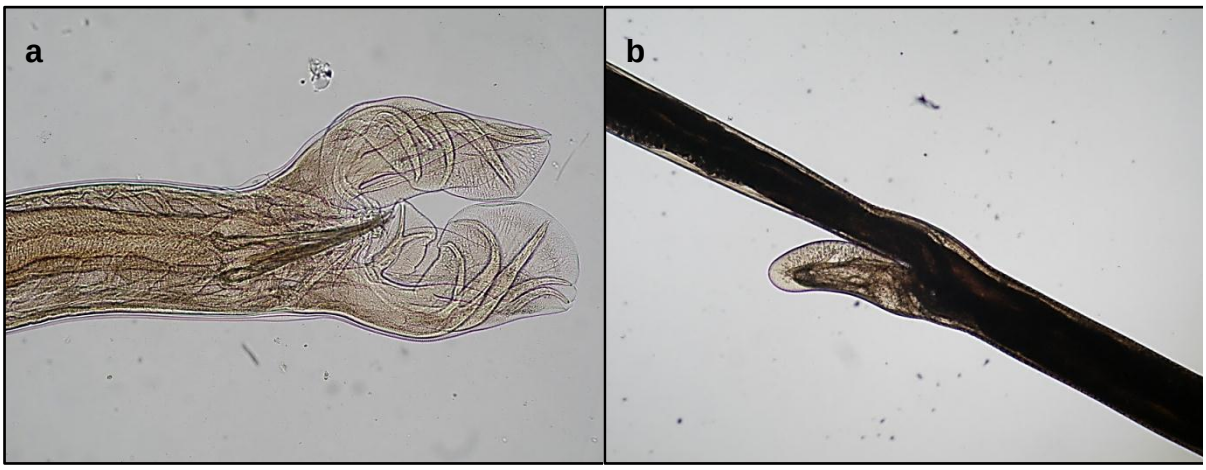

Figure 3.5: Male (a) and female (b) adult of *H. contortus*.

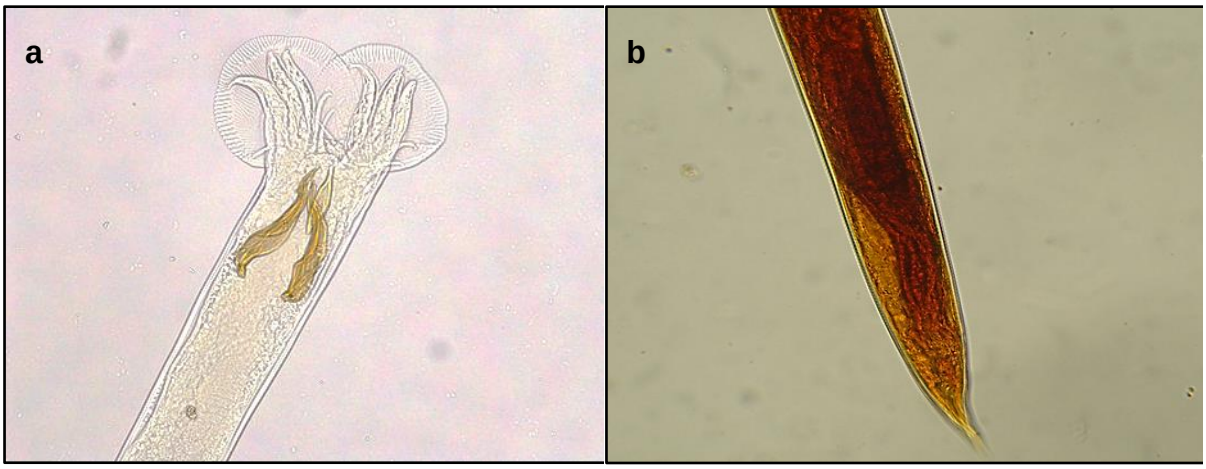

Figure 3.6: Male (a) and female (b) adults of *T. colubriformis*.