DETERMINING THE IMPACT OF PROTOZOAN AND STRONGYLID PARASITES ON MEAT LAMB PRODUCTIVITY

UTILISING MOLECULAR DIAGNOSTIC METHODS FOR THE DETECTION OF INTERNAL PARASITES IN LAMBS

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ABSTRACT

Internal parasites (strongylid gastrointestinal helminths) have been reported to decrease lamb productivity in extensive grazing sheep enterprises. Increased interest into intestinal, protozoan parasites; Cryptosporidium and Giardia, has arisen due to their potential public health risks. Little research has examined their prevalence and impact on productivity in extensively managed livestock. Despite molecular diagnostic techniques having the capability to facilitate rapid identification, improve control and enhance prevention strategies for disease pathogens, little investigation has been conducted to compare molecular tests with traditional diagnostic methods.

Longitudinal studies observed that 47–81% of lambs sampled, tested positive for Cryptosporidium or Giardia at least once in their lives over five sampling occasions. Cryptosporidium xiaoi and G. duodenalis assemblage E were the most common species/genotypes isolated from Pingelly (Farm A) and Arthur River (Farm B). Zoonotic species/genotypes were also isolated but in low numbers. Cryptosporidium xiaoi was isolated on two occasions from dam water on Arthur River, while C. ubiquitum and G. duodenalis assemblage E were detected in dam water from Frankland. A novel, possibly new genotype (sheep genotype I) was identified in six Cryptosporidium isolates from Arthur River. Cryptosporidium parvum and C. ubiquitum were the most common species detected in Boyup Brook and Kojonup flocks.

Statistical analyses revealed lambs positive for Cryptosporidium on at least one sampling occasion had lighter HCWs and lower dressing percentages when compared to lambs never positive for Cryptosporidium for Farms A and B, respectively. On Farm B,
lambs positive for *Giardia* on at least one occasion had lighter HCWs and lower dressing
percentages when compared to lambs never positive for *Giardia*. *Cryptosporidium*-positive
lambs at the second sampling were 3.84–4.72 times more likely to have non-pelleted
faeces (faecal consistency score [FCS] ≥ 3), when compared to *Cryptosporidium*-negative
lambs for Farms A and B. Lambs on Boyup Brook and Kojonup farms that were positive for
*Cryptosporidium, Giardia* or both, were 2.4–14.0 times more likely to have non-pelleted
faeces. Furthermore, a higher number of internal parasites detected per lamb was
associated with lower body condition score (BCS) and higher FCS on the Boyup Brook and
Kojonup farms. *Cryptosporidium*-positive lambs were 3.36–2.96 times more likely to have
moderate to severe breech fleece faecal soiling scores (3 – 5), when compared to
*Cryptosporidium*-negative lambs at the second sampling for Farms A and B. Live weight,
growth rate and BCS were inconsistently associated with protozoa detection across
different samplings and farms.

A further study compared the performances of two lamb flocks exposed to different
natural strongylid larval challenges. A new innovative, molecular approach was developed
to recover strongylid larvae from pasture, which had a strong, negative correlation
($r^2=0.91–0.95$) with pasture larval counts used to detect and quantify strongylid larvae
species on pasture. Flock L (exposed to a low larval challenge) had greater dressing
percentages greater than Flock S (exposed to a higher larvae challenge). Within flock
analyses of the Frankland flocks found lambs positive for *Giardia* at least once had lighter
HCWs and lower dressing percentages, when compared to lambs never positive for
*Giardia*. 

ABSTRACT
A written questionnaire which surveyed 139 (41.4%) meat lamb enterprise owners/managers in southern Western Australia, found evidence of diarrhoea was reported on 64.8% of farms. A binary logistic regression analysis revealed that the source of livestock drinking water was associated with the incidence of diarrhoea. Lamb flocks that sourced water from a dam, were 117 times more likely to have active or recent evidence of diarrhoea. Overall, 10.1% and 14.4% of respondents were aware of Cryptosporidium and Giardia, respectively.

Comparison between a molecular diagnostic technique (identifying strongylid species by screening genomic DNA extracted directly from faeces) and the traditional McMaster WEC method, found high levels of agreement (kappa statistic ≥0.93) between the test results for detecting patent strongylid infections in two separate epidemiological studies. The findings that some lambs tested negative for strongylid infections while grazing pastures known to be infested with larvae, together with the strong correlations between WEC and the number of strongylid species detected per lamb, both suggest that strongylid eggs are the likely main source of strongylid DNA.

The findings of this thesis suggest that molecular identification of internal parasites is potentially negatively associated with phenotypic performance traits of lambs. Protozoa-positive lambs had reduced production performances (lighter carcase weights and reduced dressing percentage), when compared to protozoa-negative lambs. For such molecular techniques as that were employed in this research to be introduced into routine veterinary diagnostics, they need to: (1) quantify the magnitude of infections, (2) provide cost-benefits to sheep producers, (3) display consistent associations/correlations with phenotypic performance traits of livestock and (4) be cost-beneficial for diagnostic laboratories to
conduct (sales volume and equipment costs). The future development of multiplex, real-time, quantitative PCR (qPCR) assays capable of detecting and quantifying multiple pathogen infections (parasites and bacteria) in a single assay, would facilitate the uptake of such tests for both veterinary and human diagnostics.
DECLARATION

I declare this thesis is my own account of my research and contains as its main content, work which has not been previously submitted for any degree and is not currently being submitted for any other degree or qualification. I declare that I have conducted the research described except where otherwise acknowledged.

...........................................

Signature

Joshua Paul Alexander Sweeny

April 2012
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PUBLICATIONS

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J.P.A Sweeny, C. Jacobson, I. Robertson and U.M. Ryan. 2011. Prevalence and on-farm risk factors for diarrhoea in meat lamb flocks in Western Australia. *21st Annual Combined Biological Sciences Meeting*, University of Western Australia, Perth, Western Australia, Australia. pg 80.


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The Murdoch University Veterinary Trust Sandrina Park Post Graduate Award, Murdoch University, 2009. J.P.A Sweeny. 2009. Determining the impact that protozoan and strongylid parasites have on meat lamb productivity.


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in lambs using faecal DNA extractions. 21st Annual Combined Biological Sciences Meeting, University of Western Australia, Perth, Western Australia, Australia. pg 49.

SYMBOLS

~:           approximately

β:           beta

°C:          degrees Celsius

=:           equals

>:           greater than

≥:           greater than or equal to

κ:           kappa statistic

<:           less than

≤:           less than or equal to

µ:           micro

ī:           naive
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<tr>
<td>ABARE:</td>
<td>Australian Bureau of Agricultural and Resource Economics</td>
</tr>
<tr>
<td><em>ad libitum</em></td>
<td>freely available (Latin)</td>
</tr>
<tr>
<td>ANOVA:</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AQIS:</td>
<td>Australian Quarantine and Inspective Services</td>
</tr>
<tr>
<td>AUD:</td>
<td>Australian dollars</td>
</tr>
<tr>
<td>BZ:</td>
<td>benzimidazole</td>
</tr>
<tr>
<td>C. <em>jejuni</em>:</td>
<td><em>Campylobacter jejuni</em></td>
</tr>
<tr>
<td>C. <em>ovina</em>:</td>
<td><em>Chabertia ovina</em></td>
</tr>
<tr>
<td>CI:</td>
<td>confidence interval</td>
</tr>
<tr>
<td>CMI:</td>
<td>cell mediated immunity</td>
</tr>
<tr>
<td><em>Cp. pecorum</em>:</td>
<td><em>Chlamydophila pecorum</em></td>
</tr>
<tr>
<td>C_q:</td>
<td>cycle number at which the fluorescence threshold was exceeded</td>
</tr>
<tr>
<td>DM:</td>
<td>dry matter</td>
</tr>
<tr>
<td>DM%:</td>
<td>dry matter percentage</td>
</tr>
<tr>
<td>DMI:</td>
<td>dry matter intake</td>
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<tr>
<td>DNA:</td>
<td>deoxyribonucleic acid</td>
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Page xviii  ABBREVIATIONS
dNTP: Deoxyribonucleotide triphosphate

DSE: dry sheep equivalent

E. coli: Escherichia coli

ELISA: enzyme-linked immunosorbent assay

epg: eggs per gram

et al. and others (Latin et alii)

FCS: faecal consistency score

FDM%: faecal dry matter percentage

FECRT: faecal (worm) egg count reduction test

FOO: feed on offer

g: gram

g: unit

H. contortus: Haemonchus contortus

HCW: hot carcase weight

hr: hour(s)

kg: kilogram

km: kilometres

L: litres

ABBREVIATIONS
L₁: first stage larvae
L₂: second stage larvae
L₃: third stage larvae
L₄: fourth stage larvae
L₅: fifth stage adult larvae
LV: levamisole
mRNA: mitochondrial ribonucleic acid
mg: milligram
MgCl₂: Magnesium Chloride
min: minute(s)
mL: millilitre
ML: marcocyclic lactone
MLA: Meat and Livestock Australia
mm: millimetres
mM: milli molar
n/a: not applicable
n/s: not significant
OP: organophosphate
OJD: Ovine Johne's disease
pg: picogram
pH: negative log of hydrogen ion concentration
PETA: People for the Ethical Treatment of Animals
PCR: polymerase chain reaction
PPRI: peri-parturient relaxation of immunity
qPCR: quantitative real-time PCR
rDNA: ribosomal deoxyribonucleic acid
$r^2$: linear regression correlation coefficient
RNA: Ribonucleic acid
rRNA: ribosomal ribonucleic acid
s: second(s)
S.E.M.: standard error of the mean
S.E.D.: standard error of the difference
swt: shipped weight
spp.: species
*T. circumcincta*: *Teladorsagia circumcincta*
U: units
UK: United Kingdom
µM: micromolar
USA: United States of America
WA: Western Australia
WEC: faecal worm egg count
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CHAPTER 1: INTRODUCTION

The Australian domestic livestock industry is a major component of Australian agriculture, contributing significantly to the nation’s export value and providing vast employment opportunities in both city and regional areas. The red meat livestock industry encompasses the production and processing of sheep, cattle, and goats for international export and also for domestic consumption. An Australian Bureau of Agricultural and Resource Economics (ABARE) report calculated the value of this industry to be $15.8 billion Australian Dollars (AUD), with sheep and lamb livestock contributing $AUD4.0 billion (ABARE, 2010; Athas, 2011).

1.1 SHEEP AND LAMB INDUSTRY IN AUSTRALIA

Australia is the world’s second largest exporter of sheep meat after New Zealand and although the Australian dollar is at a high level, both lamb exports and overseas demand have increased and are forecast to rise further (ABARE, 2010; Athas, 2011). The majority of Australia’s 67.7 million sheep (as of June 2010) are located in the regional areas of New South Wales (34%), Western Australia (WA) (23%) and Victoria (22%) (ABARE, 2010; Athas, 2011). In WA, where ~14.4 million sheep are located on farms in southern areas of the state, there is a Mediterranean environment (hot, dry summers and cool, wet winters) with a predominantly winter rainfall pattern (Hill et al., 2004; Moeller et al., 2008). The WA flock is expected to fall by 15% to 12.4 million, due to severe drought conditions experienced by southern WA (particularly in 2010), before re-building from 2012 onwards (Athas, 2011). The major pathogenic diseases in southern Australian sheep enterprises include: internal parasites, blowfly strike and lice, with internal parasites
(strongylid worms only) documented as causing the greatest production losses in sheep enterprises (McLeod, 1995; Sackett et al., 2006).

1.2 INTERNAL PARASITES IN SOUTHERN AUSTRALIAN FLOCKS

Strongylid worms are the major group of internal parasites in southern Australia that impact upon both sheep enterprise productivity and profitability. The major strongylid species reported in southern Australian flocks include Teladorsagia circumcincta, Trichostrongylus spp., Haemonchus contortus, Chabertia ovina, Oesophagostomum spp. and Nematodirus spp. (Besier and Love, 2003). Their pathophysiology and associations with production losses are discussed in the Literature Review (Chapter Two).

1.3 THE ECONOMIC IMPACT OF STRONGYLIID WORMS ON SHEEP ENTERPRISE PRODUCTION IN SOUTHERN AUSTRALIA

In an animal health and welfare report by Sackett et al. (2006), internal parasites were documented to reduce wool staple strength (Barger and Southcott, 1975b), wool fibre diameter and wool growth (Pullman et al., 1991; Larsen et al., 1995; Simpson, 2000) and also to reduce lamb live weights and growth rates (Pullman et al., 1991; McClure et al., 1999; Liu et al., 2005; Louie et al., 2007). Reduced ewe fertility, reduced lambing percentage and increased mortality rates have also been reported (McClure, 2000; Vagenas et al., 2007). The total national economic loss of income (losses in income, combined with treatment and control costs), attributed to internal parasites (strongylid worms) in this report was calculated to be $AUD369 million (Sackett et al., 2006).
Diarrhoea in lambs is typically associated with their immune-mediated responses towards strongyloid worm infection and development, whereby fluid absorption from the gastrointestinal tract is disrupted. However, diarrhoea is a complex, multi-factorial condition, with infectious agents (parasites, bacteria and viruses) and non-infectious agents (nutrition, fungi and environmental stresses) all reported as contributing factors (Larsen et al., 1994; Skirrow, 1994; Eerens et al., 1998; Larsen et al., 1999; Broughan and Wall, 2007; Belloy et al., 2009; Jacobson et al., 2009b; Williams et al., 2010b). Diarrhoea is a significant management challenge for sheep enterprises and has been reported to be associated with reduced growth rates (Green et al., 1998) and increased risk of breech fleece faecal soiling (commonly referred to as dag, where faeces adhere to the breech of the lambs and sheep; Figure 1.1) (Larsen et al., 1999; Broughan and Wall, 2007).

Figure 1.1: Merino sheep showing varying degrees of breech fleece faecal soiling at the breech (‘perianal’) area.

Both diarrhoea and breech fleece faecal soiling are major risk factors for cutaneous blowfly myiasis (typically referred to as ‘blowfly strike’) (Morley et al., 1976; Hall and Wall,
1995; Broughan and Wall, 2007). Blowfly strike remains the most prevalent ectoparasitic mediated disease of domestic sheep in sheep-rearing countries worldwide and is also an important welfare issue (Wardhaugh and Morton, 1990; Hall and Wall, 1995; Ward, 2001a; Snoep et al., 2002; Bisdorff and Wall, 2008). Furthermore, both diarrhoea and breech fleece faecal soiling in lambs awaiting slaughter increases the risk of carcase contamination by enteric microbes that are associated with meat spoilage and human food poisoning (Greer et al., 1983; Biss and Hathaway, 1996; Hadley et al., 1997; Garcia et al., 2010). In addition, faecal contamination of carcases is associated with trimming of effected carcase tissue, which in turn limits abattoir productivity (Hadley et al., 1997; Gill et al., 1998b).

1.3 RECENT ADVANCES IN UNDERSTANDING PROTOZOA IN SHEEP

Recent investigation into internal parasites other than the strongylid worms in lambs, has found that protozoan parasites potentially may play an important role in both diarrhoea and production loss. Protozoa have been reported at varying prevalences (3 – 82%) in sheep worldwide (Geurden et al., 2008b; Ozdal et al., 2009; Paoletti et al., 2009; Yang et al., 2009; Robertson et al., 2010; Wang et al., 2010b; Fiuza et al., 2011). The important species reported in sheep in WA include Cryptosporidium, Giardia and Eimeria (Yang et al., 2009), although their associations with lamb production remain unclear.

Unlike the strongylid worms, Cryptosporidium and Giardia are zoonotic parasites, whereby when passed in the faeces of both domestic livestock (Bodley-Tickell et al., 2002; Yang et al., 2009; Smith et al., 2010) and wildlife (marsupials and feral animals) (Power et al., 2003; Power et al., 2004), they have the potential to contaminate water used for recreation, drinking, swimming or food preparation (Medema et al., 1998; Bodley-Tickell et
It is possible for human infections to occur from water contamination and these are discussed, along with life cycles, pathophysiology and treatments in the Literature Review (Chapter Two). The associations that protozoa have with livestock productivity remains unclear, with no studies investigating their impacts on lamb productivity, whereby lambs extensively grazing pastures naturally acquire infections.

1.4 THESIS OUTLINE

The broad aim of this thesis is therefore to investigate lamb production to ascertain the prevalence of protozoan and strongylid parasites and to determine whether they are associated with decreased productivity performances in meat lambs (raised specifically for slaughter) in WA. Molecular diagnostic techniques will be utilised to determine internal parasite species. Single or multiple parasite interactions will be examined to determine if any are associated with carcase weight, live weight, growth rate, body condition score (BCS), faecal consistency score (FCS) and faecal dry matter percentage (FDM%). If protozoa are negatively associated with production or faecal attributes, farm management control practices will be recommended to help sheep enterprises minimise their impacts on lamb production.

This research aiming to help determine what the impact internal parasites have on lamb meat production in southern Australia, has relevance for parasitologists, veterinarians, sheep consultants, managers of sheep enterprises and those who work in Australian Quarantine Inspection Services.
CHAPTER 2: LITERATURE REVIEW

INTERNAL PATHOGENS IN WESTERN AUSTRALIA ASSOCIATED WITH PRODUCTION LOSSES AND DIARRHOEA IN LAMBS

Internal parasites are a significant obstacle that WA sheep enterprises encounter whilst striving for maximum profits from their sheep enterprises. The first section of this literature review describes the sheep and lamb industry in WA, the industry’s importance and flock changes associated with shifting consumer demands. Thereafter, follows a discussion which examines those internal parasites associated with lamb production and diarrhoea in WA. A focus is directed towards strongylid nematodes and protozoa (describing their taxonomy, life cycles, clinical signs, pathophysiology, production consequences, diagnosis, treatment and control methods). The final section provides detail of the non-pathogenic causes of production losses and diarrhoea in WA lambs.

2.1 WESTERN AUSTRALIAN SHEEP AND LAMB PRODUCTION

2.1.1 GENERAL INDUSTRY OVERVIEW

The geographical area in WA where sheep production occurs, encompasses most of the wheat sheep zone of WA, with the bulk of those sheep and lambs produced, predominantly either sold for live export or domestic slaughter. Persistent dry conditions across WA have been a contributing factor to the recent flock decline; falling to ~14.4 million sheep (Athas, 2011). Lamb producers in WA have the following options when selling their lambs; live export, sell to other sheep enterprises/breeders or slaughter at
commercial abattoirs (Fletcher et al., 2009; ABARE, 2010). Between 2010 and 2011, it has been reported that producers selling lambs for live export received a price of $AUD59 per head, which was 22% less than that received for lambs sold directly for commercial slaughter (ABARE, 2010; Athas, 2011). High lamb prices ($AUD5.02–5.13/kg of carcase weight) and increased demand for lamb meat products, has therefore resulted in a shift towards meat lamb production in sheep enterprises in WA, whereby producers sell a greater percentage of their lambs for slaughter at local abattoirs, with high carcase prices providing an opportunity to maximise their profits (Athas, 2011). The value of those sheep and lambs sold for slaughter accounts for the large majority of WA’s total value of lamb and sheep production.

With many WA extensive (broad-acre) farming properties operating diverse enterprises (managing sheep for both meat and wool products, cattle for meat and dairy products, in addition to cereal crop production), the majority of Western Australian sheep enterprises produce large quantities of grain (wheat, oats, lupins, canola and barley). This gives farmers the option to utilise some of their harvested grain as supplementary feed for grazing livestock, particularly in drier months when feed is scarce. Such practice is typical for meat lamb enterprises and is associated with those enterprises where a higher proportion of lambs are sold directly for local slaughter. In addition, lambs supplemented on grain generated a real net cash income per hectare of $64/year, ~3 times more than for non-grain supplementing enterprises (Fletcher et al., 2009; ABARE, 2010).

2.1.2 TRENDS IN SHEEP AND LAMB PRODUCTION

Over the past two decades, Australian sheep enterprises have responded to the market signals by shifting the focus of their flock from wool to lamb meat production.
As a result, the flock demographics of Australian sheep enterprises have changed following an increase in both the numbers of lambs slaughtered and their carcase weights (Fletcher et al., 2009; ABARE, 2010; Athas, 2011). An increase in demand for lamb meat has resulted in a rise in production by 37% and the quantity of lamb meat exported has risen by 92% over the past 10 years (Fletcher et al., 2009; ABARE, 2010), with a further 7% forecast increase in production to 436,000 tonnes shipped weight (swt) by the end of 2011 (Athas, 2011).

2.1.1.1 Lamb production, carcase price, consumer expenditure and export

The combination of record high lamb prices, depressed wool prices and an increased domestic and international market demand for lambs and lamb products, have encouraged many sheep enterprises to either commence lamb for slaughter lamb or to expand and increase their existing lamb meat production (Figure 2.1) (ABARE, 2010; Athas, 2011). However, these moves to expand production have been adversely affected by below average rainfall in recent years (ABARE, 2010; Australian Bureau of Meteorology, 2011b). Hotter and drier than normal conditions in southern Australia have limited both on-farm feed availability and contributed to reduced flock numbers (Athas, 2011). Strong growth in demand for lamb products both domestically and internationally, when combined with reduced sheep numbers and adverse seasonal conditions, has resulted in real lamb commodity prices rising sharply over the past two decades (Figure 2.2) (ABARE 2010).
Figure 2.1: Australian lamb production (in thousands of tonnes of carcase weight) and average lamb carcase weight (kg/lamb) (Athas 2011).

Figure 2.2: Index of real commodity prices for lamb, beef, wheat and wool in Australia (ABARE 2010).

Australian domestic lamb consumption reached 220,000 tonnes in 2010, despite limited supply, record market prices, interest rate rises and strong competition from...
international markets (Figure 2.3). Average retail lamb prices have risen by 5% to $AUD14.55/kg, due to a rise in demand for lamb (domestically and internationally), combined with reduced supplies and high saleyard prices (Athas, 2011). Domestic lamb consumption is expected to rise to 273,000 tonnes by 2015, with the average consumption per person rising ~25% to 11.4kg/year (Athas, 2011).

Australian lamb exports reached 155,496 tonnes shipped weight (swt) in 2010, which was the third highest year on record, with the Middle East our largest export market, followed by the United States of America (USA), China/Hong Kong, Europe and South-East Asia (ABARE, 2010; Athas, 2011) (Figure 2.3). A total of 37,670 tonnes swt was exported to the Middle East in 2010 and this was the first year Australian lamb exports to that region surpassed those to the USA (Athas, 2011).

**Figure 2.3:** Left – Domestic lamb consumption (in thousands of tonnes of carcase weight) and retail market price (average cents/kg retail weight). Right – Australian lamb meat exports (in thousands of tonnes of shipped weight) and percentage of lamb production (Athas 2011).
2.1.1.2 Number of meat lamb farms and changing flock demographics

There has been a 21% increase in the number of sheep enterprises that sold lambs for commercial slaughter between 1990–2010 (ABARE, 2010; Athas, 2011). This decision by sheep enterprises to place a greater focus on meat lamb production has resulted in changes to both flock demographics and management. Producers have increased their enterprises’ specialisation in meat lamb production by increasing the female (ewe) proportion of their entire sheep flock, to maximise lamb numbers and decrease the proportion of wethers (Figure 2.4). This is reinforced by both the increased number of ewes mated and lambs weaned in 2009–10 (ABARE, 2010; Athas, 2011). A further advantage of incorporating more meat production into sheep enterprises, was that enterprises which managed meat breeds, were reported to have a higher chance of twin births and greater numbers of lambs weaned (ABARE, 2010).

Figure 2.4: Demographic changes of the Australian sheep flock (ABARE 2010).
2.2 THE ECONOMIC IMPACT OF INTERNAL PARASITES ON THE
AUSTRALIAN SHEEP INDUSTRY

Internal parasites are a major concern to the Australian sheep industry, with an alarming increase in anthelmintic resistance making their management and control difficult (Besier and Love, 2003; Coles et al., 2006; Woodgate and Besier, 2010). Blowfly strike, lice and internal parasites are the most important diseases that affect sheep enterprises, with the greatest losses in production attributed to internal parasites (Figure 2.5) (McLeod, 1995; Sackett et al., 2006). In 1994, internal parasites were calculated to cost the Australian sheep industry $AUD220 million (McLeod, 1995). This was made up of: $AUD80 million for control, $AUD40 million from mortalities and $AUD100 million through loss of income (reduced production performance).

A more recent study by Sackett et al., (2006) for Meat and Livestock Australia (MLA), examined the economic cost of endemic diseases on Australian sheep and cattle profitability, along with the disease impacts on animal health and welfare. Sackett et al., (2006) evaluated the national cost of internal parasites to the Australian sheep industry at $AUD369 million; $AUD59 million in control costs and $AUD310 million attributed to loss of income. This equated to 8.7% of the total value of the Australian sheep industry in 2006. The control expenses were due to the costs associated with anthelmintic treatment and also for labour as required with the crutching process. However a limitation of that study was that strongylid nematodes were the only genera of internal parasites included in this research.

For meat (prime) lamb (Merino ewes joined with meat breed rams to produce crossbred lambs raised specifically for slaughter) enterprises that utilised internal parasite
control programs, Sackett et al., (2006) quoted the internal parasite losses to be $AUD4.93/sheep. However for those enterprises with poor or no control programs, the losses per head reached as high as $AUD12.08 per head. The reduced income due to internal parasitism in the winter rainfall zones was $AUD4.61 per head for sheep enterprises and $AUD7.75 per head for specialised meat lamb enterprises. In addition to $AUD4.61 per head reduced income, increased sheep husbandry expenses added an extra $AUD0.12 per head for crutching and $AUD0.71 per head for anthelmintic treatments (Sackett et al., 2006).

![Figure 2.5: National cost of endemic diseases to the sheep industry; control costs (expenses) and production losses (reduced income) (Sackett et al., 2006).](image)
Sheep, particularly those lambs exposed to strongylid larvae on pastures over winter and spring periods, require ongoing monitoring and possible anthelmintic treatment to minimise production losses, reduce mortality risks and to maintain their overall health and welfare. In a range of Western Australian locations, groups of anthelmintic treatments are non-effective due to the proportions of worm populations having resistance to a variety of different anthelmintic treatment groups (Besier and Love, 2003; Besier, 2007; Woodgate and Besier, 2010). Apart from the new anthelmintic treatment monepantel, no other anthelmintic chemicals are capable of targeting both resistant and susceptible strongylid worms (Hosking et al., 2009; Sager et al., 2009). Anthelmintic resistance is discussed in more depth in “section 2.4.5”.

2.2.1 DIARRHOEA AND BLOWFLY STRIKE

Diarrhoea and breech fleece faecal soiling are important risk factors associated with the development of flystrike (blowfly strike), commonly observed in the perianal breech area of sheep (Morley et al., 1976; French et al., 1994; Hall and Wall, 1995; Leathwick and Atkinson, 1995; Broughan and Wall, 2007). Ovine cutaneous myiasis (blowfly strike) remains the most prevalent ectoparasite disease for the majority of sheep enterprises worldwide (Hall and Wall, 1995) including Australia. Blowfly strike is caused by the carrion-feeding habits of flies from the Calliphoridae family, with flies that are found in Australia; Lucillia spp. Calliphora spp. and Phormia spp., being attracted to faecal and urine soiled areas around the breech area of sheep (Zumpt, 1965).

Lucillia cuprina is the primary fly causing the initial blowfly strike in Australia sheep (Ward, 2001a, b). Female L. cuprina flies deposit their eggs or live larvae on the faecal and urine stained areas located at the breech area of effected sheep (Blood and Studdert,
1988; Hall and Wall, 1995; Broughan and Wall, 2007). The hatched maggots cause destruction of the skin and secondary strikes by *Chrysomya* spp. (hairy blowfly), resulting in hairy blowfly maggots burrowing into and destroying skin and muscle tissue. As the size of the faecal and urine stained area increases, so does the risk of sheep suffering blowfly strike and this is the critical risk factor which predisposes sheep to the disease (Morley *et al.*, 1976; Watts *et al.*, 1979; Leathwick and Atkinson, 1996; Heath and Bishop, 2006). Blowfly strike is endemic in the Australian sheep industry, causing economic losses associated with; reduced production, increased chemical treatment expenses and increased mortalities (McLeod, 1995; Sackett *et al.*, 2006; Scobie *et al.*, 2008). The condition holds strong welfare concerns for both members of the public and sheep producers.

### 2.3 INFECTIOUS PATHOGENS IN WESTERN AUSTRALIA ASSOCIATED WITH PRODUCTION LOSSES AND DIARRHOEA IN LAMBS

As both lamb production and the number of lambs for slaughter enterprises increase, it is important that these enterprises maximise their profits from those meat lambs sent for commercial slaughter. There are a number of infectious and non-infectious factors that are important for lamb enterprises in WA. The major pathogens which are responsible for production losses associated with reduced growth rates and the occurrence of diarrhoea in lambs, includes strongylid nematodes, protozoa, bacteria and viruses. It is important to understand and quantify the economic losses attributed to infectious pathogens, with the following sections of this literature review concentrating predominantly on two specific internal parasites; strongylid nematodes and protozoa.
2.4 STRONGYLID NEMATODE INFECTIONS

Modern farming practices including; the development of fertiliser topdressing, introduction of legume based pastures along with improved preventive animal health control programmes, have all contributed to increased stocking rates in extensive, broad-acre grazing enterprises (Thamsborg et al., 1996; Fletcher et al., 2009; ABARE, 2010). As a result, this increased stocking density has accelerated both transmission and the pathophysiological consequences of strongylid nematodes occurring in sheep flocks, particularly in those regions where favourable climatic conditions exist for third stage larvae (L₃) transmission from pastures to sheep. Strongylid taxonomy, life cycle, impacts on production and anthelmintic resistance, are all discussed in this section.

2.4.1 TAXONOMY

The Trichostrongylidae family of nematode worms are medium in size, similar to those other worms of the Strongylidea Order. These worms have a reduced buccal capsule, thorn like cervical papillae and a well developed copulatory bursa (Gibbons and Khalil, 1982; Durette-Desset, 1983; Lichtenfels and Hoberg, 1993). The majority of worms from this family infect the gastrointestinal tract of their vertebrate host, with their eggs having near identical morphology to one another when viewed under microscopy (Whitlock, 1948; Whitlock, 1959; Animal Health Laboratories, 2005c). Therefore recovery and identification of different strongylid worm species is performed by larval differentiation, with the eggs cultured to L₃ (Lyndal-Murphy, 1993; Animal Health Laboratories, 2005a).

2.4.1.1 Major strongylid sheep worms in Western Australia

In regions of Australia that receive substantial winter rainfall, strongylid nematode infections are the most commonly implicated cause of ill-thrift and diarrhoea (Besier, 2004;
Jacobson et al., 2009b). There are three main species that are the most important parasites, from a clinical and economic perspective, which infect sheep in southern WA. These are: *Trichostrongylus* spp. (the Black Scour worm) and *Teladorsagia circumcincta* (Small Brown Stomach worm) and *Haemonchus contortus* (Barber’s Pole worm) (Eady et al., 1996; Besier and Love, 2003; Woodgate and Besier, 2010). Other less economically important species include *Nematodirus* spp., *Cooperia* spp., *Oesophagostomum* spp. and *Chabertia ovina* (Table 2.1). Usually these minor strongylid worm species are rarely present in sufficient numbers to cause severe production losses or diarrhoea in their own right, but are more pathogenic when present as part of a mixed strongylid infection (Besier and Love, 2003; Woodgate and Besier, 2010).
Table 2.1: Taxonomic and genus species of strongylidea worms in sheep (Lichtenfels and Hoberg, 1993).

<table>
<thead>
<tr>
<th>CLASS NEMATODE</th>
<th>Order</th>
<th>Family</th>
<th>Sub family</th>
<th>Genus species</th>
<th>Common name</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>STRONGYLIDEA</td>
<td>Cyathostomae</td>
<td>Oesophagostominae</td>
<td></td>
<td>Oesophagostomum</td>
<td>nodule worm</td>
<td>large intestine</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>columbianum</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Oesophagostomum</td>
<td>large bowel worm</td>
<td>large intestine</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>venulosum</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Chabertia ovina</td>
<td>large mouthed bowel worm</td>
<td>large intestine</td>
</tr>
<tr>
<td>Trichostrongylidae</td>
<td>Trichostrongylinae</td>
<td>Trichostrongylus axei</td>
<td></td>
<td>stomach hair worm</td>
<td>abomasum</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Trichostrongylus vitrinus</td>
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<td>small intestine</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Trichostrongylus colubriformis</td>
<td>black scour worm</td>
<td>small intestine</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Trichostrongylus extenuatus</td>
<td></td>
<td>abomasum</td>
</tr>
<tr>
<td>Cooperia curticei</td>
<td></td>
<td></td>
<td></td>
<td>small intestinal worm</td>
<td>small intestine</td>
<td></td>
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<tr>
<td>Cooperia oncophora</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>small intestine</td>
<td></td>
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<tr>
<td>Marshallagia marshall</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>abomasum</td>
<td></td>
</tr>
<tr>
<td>Teladorsagia circumcincta</td>
<td></td>
<td></td>
<td></td>
<td>small brown stomach worm</td>
<td>abomasum</td>
<td></td>
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<tr>
<td>Ostertagia ostertagi</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>abomasum</td>
<td></td>
</tr>
<tr>
<td>Marshallagia occidentalis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>abomasum</td>
<td></td>
</tr>
<tr>
<td>Haemonchinae</td>
<td>Haemonchus contortus</td>
<td></td>
<td></td>
<td>barbers pole worm</td>
<td>abomasum</td>
<td></td>
</tr>
<tr>
<td>Nematodirinae</td>
<td>Nematodirus filicollis</td>
<td></td>
<td></td>
<td></td>
<td>small intestine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nematodirus spathiger</td>
<td></td>
<td></td>
<td>thin necked intestinal worm</td>
<td>small intestine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nematodirus battus</td>
<td></td>
<td></td>
<td></td>
<td>small intestine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nematodirus helveticus</td>
<td></td>
<td></td>
<td></td>
<td>small intestine</td>
<td></td>
</tr>
</tbody>
</table>
2.4.2 LIFE CYCLE AND TRANSMISSION

Life cycles of strongylid worms are simple and direct compared to those of the trematodes and cestodes (Figure 2.6) (Thomas, 1982). Adult worms range from 4 – 30mm in length, depending on the sex and species of the worm, with each species establishing infections within a specific niche location in sheep (for instance *H. contortus* in the abomasum). Once the mature adults reach this niche location, they mate and the females lay their eggs. These unembryonated eggs move through the gastrointestinal tract and are passed in the faeces onto pastures by infected sheep. In the external environment, eggs embryonate and develop into first (L₁), then second stage larvae (L₂) within the faeces. The L₂ larvae feed and increase in size before moulting to L₃, but retain their L₂ cuticle as a sheath for protection against environmental extremes (Besier and Gardner, 2005; Woodgate, 2005). Infective L₃ migrate actively from faeces, provided that adequate moisture is present, onto vegetation (pasture and plants) and soil (Banks *et al.*, 1990; Tembely, 1998; Moss and Bray, 2006; O’Connor *et al.*, 2006). With most L₃ located within a range of ~25mm above ground on pasture, the L₃ infective stage is consumed by a definitive host (sheep) and the larvae mature to become fourth stage larvae (L₄). By the time they reach fifth stage adult larvae (L₅), the worms have reached their final niche location within sheep (for instance the abomasum or small intestine, depending on the species) and the cycle restarts with the laying of unembryonated eggs by female adults (Figure 2.6) (Besier and Gardner, 2005; Woodgate, 2005).
Figure 2.6: Life cycles of strongylid nematodes (Woodgate, 2005; Hobbs et al., 2007).

The development of L₄ and L₅ within sheep after ingestion of the infective L₃ occurs in ~2–3 weeks. Southern WA experiences a Mediterranean environment with hot dry summers and cool wet winters (Hill et al., 2004; Moeller et al., 2008). The overall development of these strongylids in this environment depends on climatic conditions and the specific worm species (Dobson et al., 1990b; Besier and Dunsmore, 1993a, b; Dobson and Barnes, 1995). Third stage larvae survive on pastures with higher forage levels for prolonged periods when temperature and moisture conditions are favourable (Marley et al., 2006; Moss and Bray, 2006; O’Connor et al., 2006). In winter months, some larvae may survive for as long as six months, due to higher forage levels of pasture which retain favourable moisture conditions. While in the hot, dry summer months, drier conditions contribute to larvae destruction within 1–2 months after they have been deposited from sheep or lambs (Dobson et al., 1990b; Besier and Dunsmore, 1993a; Eysker et al., 2005). During mild summers, larvae may have prolonged survival periods when protected in
faecal pellets, emerging when autumn rains bring more favourable transmission conditions (Woodgate, 2005; Moss and Bray, 2006).

2.4.2.1 The periparturient relaxation of immunity

The ability of mature ewes to maintain a solid immunity to strongylid infections is compromised during the peri-parturient period caused by a relaxation of immunity. This lowered immune status of pregnant ewes commences from ~2–3 weeks pre-partum and continues for up to ~6–8 weeks post-partum (McAnulty et al., 2001). A number of causes of this peri-parturient relaxation of immunity (PPRI) have been suggested, including endocrine-immunosuppression (Barger, 1993). However the strongest evidence suggests that PPRI is predominantly controlled by both nematode resistance of the ewe (Woolaston, 1992) and the balance between the demand and supply of metabolisable protein (Donaldson et al., 1998; Houdijk et al., 2001; Kahn et al., 2003; Houdijk, 2008).

The PPRI is commonly associated with a rise in faecal worm egg count (WEC), which is linked to a relaxation of a pregnant host restraints on female worm egg production and the subsequent establishment of infective larvae (Barger, 1993). Outside this PPRI period, mature ewes generally are capable of maintaining resistance to strongylid infections, because from ~4 weeks post-lambing, ewes have usually regained their immunity to strongylid establishment (Leathwick et al., 1999). However strongylids established during this PPRI period may remain in ewes for several weeks longer, resulting in a peak WEC ~6–8 weeks after lambing, before returning to lower levels (Brunsdon, 1971; Brunsdon and Vlassoff, 1971b, a).

The PPRI leads to both an increased contamination of pastures with strongylid worm eggs and greater numbers of infective L₃ capable of being ingested by grazing sheep
or lambs. The increased protein demand induced by lactation during pregnancy is associated with the suppression or abandonment of an established protective strongylid worm immunity (Donaldson et al., 1997). Although of greater importance, the PPRI increases the risk of naive (i) lamb infections, leading to restricted growth rates and increased mortalities, particularly in the event of H. contortus outbreaks (Besier and Dunsmore, 1993a, b; Donaldson et al., 1997).

2.4.3 CLINICAL SIGNS AND PATHOLOGY

Strongylid nematode infection relies on ingestion of infective L₃, with these larvae located on pasture stalks or near the soil surface (Callinan et al., 1982; Marley et al., 2006; Moss and Bray, 2006; O’Connor et al., 2006). Naive lambs are more susceptible to strongylid infection as they have not acquired effective immunity against strongylid worms until they reach ~6–12 months age (Smith et al., 1985; Barnes and Dobson, 1993; Stear et al., 1999).

2.4.3.1 Trichostrongylus spp. infections

Infections of Trichostrongylus spp. are commonly situated in the small intestine, but some species also reside in the abomasum. These strongylids are considered the most important species located throughout southern Australia, associated with the majority of production losses and outbreaks of diarrhoea (Woodgate and Besier, 2010). During winter months, when moisture prevails on pastures, this worm potentially causes chronic clinical signs of ill thrift and diarrhoea (Jacobson et al., 2009b). The major species of Trichostrongylus documented to be associated with these conditions in southern Australia include T. colubriformis and T. vitrinus, in addition to T. rugatus and T. axei (Beveridge et al., 1989; Bailey et al., 2009), with the latter two found in lower numbers and hence are of
less importance from a management and economic perspective. *Trichostrongylus* spp. infections in the small intestine are characterised by epithelial sloughing (McClure *et al.*, 1992), increased peristalsis and hyper-contractionility of smooth muscle (Tremain and Emery, 1994), increased fluid and electrolyte movement into the small intestine lumen, crypt cell hyperplasia, villous atrophy and epithelial tunnelling (Beveridge *et al.*, 1989; Kyriazakis *et al.*, 1996; Miller, 1996).

The role of mature villous cells is fluid absorption, while crypt cells are responsible for secretion of water and electrolytes. The combined effects of crypt cell hyperplasia, along with villous atrophy, results in reduced absorption and increased secretion in the small intestinal lumen. The net effect is greater proportions of fluid retained in the digesta as it passes through the gastrointestinal tract, as a result of the immune responses evoked by the host sheep in an attempt to expel both the mature strongylid nematode worms and larvae (Jacobson *et al.*, 2009c; Williams *et al.*, 2010c). A consequence of the above is that absorption ability in the small intestine is disrupted, leading to an increased risk of a diarrhoea outbreak (Jacobson *et al.*, 2009b; Williams *et al.*, 2010c).

### 2.4.3.2 Larval hypersensitivity scouring syndrome

Clinical infections of *Trichostrongylus* spp. potentially can result in large worm burdens, ranging from 20,000 – 30,000 per animal (Coop and Angus, 1981), which are often associated with severe diarrhoea and high WECs of up to 10,000 eggs per gram (epg) (Coop and Angus, 1981; Besier *et al.*, 2004). Another condition has been described whereby sheep that have a well developed immunity towards this species of strongylid nematode, can develop “larval hypersensitivity diarrhoea” syndrome associated with an intake of strongylid larvae from pastures (Larsen *et al*. 1999) and immature L4 numbers.
occurring within such sheep (Jacobson et al., 2009b). With a well developed immunity to these worms, WECs are typically low and this form of diarrhoea is often typically referred to as “low WEC diarrhoea” (Jacobson et al., 2009b) and caused by the defensive immune responses within the sheep or lamb (Williams et al., 2010a; Williams et al., 2010c). There are no specific tests or post-mortem findings which specifically diagnose this condition, however a recent study by Jacobson et al. (2009b) found that, while normal sheep and those sheep with diarrhoea had similar WECs, the numbers of immature Trichostrongylus spp. and T. circumcincta L₄ were much higher in sheep with diarrhoea, compared to those sheep without diarrhoea. Although dietary factors in the pasture cannot be ruled out, it is possible these L₄ potentially are a strong contributing factor towards larval hypersensitivity diarrhoea.

2.4.3.3 Teladorsagia circumcincta infections

Teladorsagia circumcincta (previously known as Ostertagia circumcincta) is one of the three most important strongylid worm species, from an economic perspective, which affect sheep in the temperate Mediterranean areas in southern Australia (Besier and Love, 2003; Woodgate and Besier, 2010), with both acute and chronic infections reported. This small brown stomach worm is a problem for sheep enterprises as it typically provides the greatest resistance to a variety of different anthelmintic treatment groups in southern Australia (Palmer et al., 2001; Besier and Love, 2003; Suter et al., 2005). Teladorsagia circumcincta infects the abomasum of sheep, with L₅ migrating from the abomasal glands to the abomasal mucosa when sexually mature. As the larvae develop, there is an associated stretching of the gastric glands and destruction of the acid-secreting cells, which are replaced by undifferentiated cells (Scott et al., 1998; Scott et al., 2000). Reduced acid secretion due to loss of these cells increases the pH of the abomasal lumen and
chyme, which leads to a reduced activation of pepsinogens (Scott et al., 1998) and an overall retardation of protein degradation in the abomasum (Coop and Angus, 1981; Fox, 1997). In cases of severe chronic infections, protein loss enteropathy occurs; whereby junctions between epithelial cells are destroyed, causing epithelial hyperplasia and increased mucosal permeability (Urquhart et al., 1996; Stear et al., 2003).

Production losses associated with T. circumcincta infections include sub-optimal growth rates, reduced feed intake, weight loss and outbreaks of diarrhoea (Liu et al., 2003; Liu et al., 2005; Louie et al., 2007; Greer et al., 2008; Kidane et al., 2009; Williams et al., 2010c). A sequential study by Scott et al. (2000) demonstrated that sheep which had T. circumcincta adult worms transported into their abomasum 2–3 days previously, displayed clinical signs that included abomasum tissue damage, protein leakage, mucosal hyperplasia, increased abomasal pH, reduced numbers of parietal cells, reduced pepsinogen activity and higher levels of inflammatory infiltrates in the abomasum. These clinical signs of T. circumcincta infections as observed by Scott et al. (2000), are also supported by Sykes and Coop (1977) and Simpson et al. (1997).

2.4.3.4 *Haemonchus contortus* infections

*Haemonchus contortus* is commonly known as the Barber’s pole worm and this blood feeding strongylid worm is found in the abomasum in sheep (Baker et al., 1959). *Haemonchus contortus* adults commence their parasitic phase in the abomasum 17–21 days after infective L₃ ingestion. As this species of strongylid is a blood sucker, the most common clinical sign associated with infections is anaemia (Clark et al., 1962; Le Jambre, 1995; Reynecke et al., 2011b, a) and this anaemia is purely haemorrhagic in character (Dargie and Allonby, 1975; Le Jambre, 1995). Adult worms have been observed to suck
blood for up to 12 mins at a time and following their detachment, the resulting haemorrhage may continue to bleed for up to 7 mins (Boughan and Hardy, 1935). Blood loss commences at least a week prior to egg laying, with the average blood loss per worm per day ~0.003mL (Dargie and Allonby, 1975) to ~0.05mL (Clark et al., 1962). Further research by Albers and Le Jambre (1983) indicated that 11 days post-infection with 10,000 *H. contortus* larvae, infected sheep were losing an average of 30mL of blood per day. This blood loss in sheep was found to have a strong correlation with worm number, worm biomass, worm egg production and WEC (Le Jambre, 1995). In addition the blood loss and anaemia associated with this parasitism contributes to a significant decreased packed cell volume (Albers and Le Jambre, 1983).

Further pathological characteristics of *H. contortus* infection include abomasal tissue damage, a rise in pH due to reduced acid secretions, decreased pepsinogen secretion (Simpson et al., 1997) and protective mucous hyperplasia (Miller, 1996). *Haemonchus contortus* is considered pathophysiologically the most dangerous strongylid species, due to the high fecundity the worm possesses and also it’s potential to rapidly contaminate pastures with a high output of eggs in sheep faeces. Furthermore, as *H. contortus* worms are blood suckers, this strongylid species can cause sudden death and contributes to high mortality rates in flocks, particularly in young lambs (Dargie and Allonby, 1975; Roberts and Swan, 1982; McClure, 2000). Although clinical signs of acute anaemia are most common, chronic infections have also been reported by Simpson (2000). Chronic burdens have been reported to cause anaemia, anorexia, reduced live weight and wool growth, depression and death (Dargie and Allonby, 1975; Simpson, 2000).
2.4.3.5 *Cooperia* and *Nematodirus* infections

*Cooperia* spp. and *Nematodirus* spp. infections are also found in the small intestine of sheep. Although clinical signs of these infections are similar to those associated with *Trichostrongylus* spp. infections, they are generally of minor significance unless part of a mixed infection (Heckendorn *et al*., 2007). *Nematodirus* spp. are erratic egg layers, and their WECs have poor correlations with actual worm burdens (McKenna, 1981). *Nematodirus* spp. infections are of minor significance with respect to their effect on live weight (Bishop *et al*., 2004; Morris *et al*., 2004). *Cooperia curticei* is a relatively uncommon strongylid worm, but may be found in some locations throughout southern Australia (Barger and Southcott, 1975a). These infections are of minor consequence to sheep productivity, unless present with another major strongylid species. Common clinical signs which are associated with infected sheep include ill-thrift and decreased growth rates (Kerboeuf *et al*., 2000; Heckendorn *et al*., 2007).

2.4.3.6 *Oesophagostomum* and *Chabertia* infections

*Oesophagostomum columbianum* (the nodule worm) is rarely seen in high numbers, as modern anthelmintic treatments have been effective in controlling and eradicating this strongylid without the development of any significant resistance challenges. Immature *Oesophagostomum* spp. larvae migrate through the small intestine to the large intestine where they mature. Adult worms cause damage to walls of the large intestine (Eysker, 1980; Roy *et al*., 2003), with the result that developing larvae in the large intestine are often encapsulated, forming nodules along the intestinal wall (Stewart and Gasbarre, 1989). Adult worms cause a thickening of the large intestine mucosa, resulting in possible decreased nutrient absorption. The damage inflicted to the walls of the large intestine often makes it no longer useful for sausage skin casings (Stewart and Gasbarre, 1989).
*Chabertia ovina* is morphologically and pathologically very similar to *Oesophagostomum* spp., as it also causes damage to the mucosal lining of the large intestine. This parasite does not form nodules in the large intestine, with ulceration and minor haemorrhaging the reported outcomes (Herd, 1971b, a; Eysker, 1980).

### 2.4.4 CONSEQUENCES FOR SHEEP PRODUCTION

The pathophysiological changes to sheep infected with a strongylid infection have been well documented and can be summarised as reduced feed intake and efficiency of nutrient utilisation (Coop *et al.*, 1977; Sykes *et al.*, 1977; Abbott *et al.*, 1986; Bown *et al.*, 1991a; Sykes and Greer, 2003) and subsequently lamb performance.

#### 2.4.4.1 Reduction in feed intake

The depression in feed consumption by sheep infected with strongylid worms is a primary mechanism by which strongylid infections reduce productivity (Coop *et al.*, 1977; Bown *et al.*, 1991a; Houdijk, 2008). Reduction in feed intake is approximately proportional to the strongylid larval intake by sheep and this observation is quite consistent for all major strongylid species (Coop and Sykes, 2002; Sykes and Greer, 2003). The availability of nutrients for the immune response in the gastrointestinal tract is determined by the supply of nutrients to the sheep from feed intake, body reserves and the demands of other physiological processes such as growth, pregnancy or lactation (Adams and Liu, 2003; Greer, 2008). Following establishment of a strongylid infection, there is a reduction in available energy due to a lowered feed intake and overall less energy for functional maintenance in infected sheep (Sykes and Greer, 2003; Greer, 2008). The remaining metabolisable energy is diverted away from growth and production of the sheep (muscle and wool growth), to provide energy towards an immune response (i.e. gastrointestinal
tract tissue and inflammatory responses) against the strongylid infection (Coop and Sykes, 2002; Adams and Liu, 2003; Liu et al., 2003). This is referred to as “nutrient partitioning” and results in a net loss of energy. The remaining energy balance is then utilised for the production performance characteristics, meat, wool and growth rate and when compared to parasite free sheep, the latter have greater energy input into their production growth (Figure 2.7) (Coop and Sykes, 2002; Adams and Liu, 2003; Liu et al., 2003; Louie et al., 2007). Nutrient partitioning is an active process under complex hormonal control that determines where the flow of available nutrients is directed.

Figure 2.7: Schematic representation indicating the effect of strongylid worms on nutrient (protein) partitioning in sheep (ME preferentially utilised by the alimentary tract for its maintenance and the local immune response as evoked by worm infections, which occurs at the expense of peripheral tissues [meat, wool, skeleton and milk]) (Coop and Sykes, 2002).
A negative correlation between growth rate and strongyle worm burden in young sheep has been documented in Merino lambs (3–9 months old) with a *T. colubriformis* infection (McClure *et al.*, 1999; Louie *et al.*, 2007). Liu *et al.*, (2005) investigated the physiological consequences of strongylid parasites on live weight and growth rate, with infected Merino rams finishing with live weights on average 5kg less than for non-infected rams. Studies on the daily nitrogen balances of sheep with intestinal and/or abomasal strongylid infections, have found that up to 36% of nitrogen leaks from the gastrointestinal tract due to damage to the gastrointestinal wall, combined with epithelial sloughing and increased mucous protein production (Poppi *et al.*, 1986; Bown *et al.*, 1991b). The nitrogen and available energy remaining in the gastrointestinal tract is directed to protein synthesis for the preferential repair of gastrointestinal tract tissue (Coop and Sykes, 2002).

**2.4.4.2 Overall effect of worm challenge on productivity of meat lambs**

The production effects of strongylids on meat lambs have not been well described in the literature, although studies in young Merinos suggest that strongylid worms contribute to reduced growth rates (McClure *et al.*, 1999; Louie *et al.*, 2007).

Liu *et al.*, (2005) investigated the physiological consequences that the strongylid worms *T. circumcincta* and *T. colubriformis* had on live weight and growth rate, with infected Merino rams finishing with a live weight on average 5kg less than for uninfected 18 month old rams. Muscle protein deposition in the carcases of *T. circumcincta* and *Trichostrongylus* spp. infected sheep have been reported to be reduced by 10% and 40% respectively, when compared to non-infected control sheep offered the same nutrition (Coop and Angus, 1981). A recent study in New Zealand found that anthelmintic resistance, specifically for albendazole, resulted in sheep having a 2.8kg reduction in live
weight and also a 2.8kg (14%) reduction in carcase weight (Sutherland et al., 2010). Jacobson et al. (2009c) also found that strongylid infection reduced dressing percentages (carcase weight as a proportion of live weight) in Merino wethers.

In addition to strongylid parasites impairing muscle deposition, low level *T. circumcincta* infections have been shown to reduce the growth of skeletal bones (Sykes et al., 1977; Coop et al., 1981) and may be a contributing factor to osteoporosis in young growing lambs. These effects are likely to arise from a restriction in available energy and protein rather than mineral deficiencies and may be important for fast-growing lambs (Coop et al., 1981).

### 2.4.5 TREATMENT, ANTHELMINTIC RESISTANCE AND CONTROL

The prevalence of anthelmintic resistance in strongylid worm populations provides an ever-growing challenge to sheep enterprises worldwide (McKellar and Jackson, 2004; Coles et al., 2006; Waller, 2006; Stafford et al., 2009; Mitchell et al., 2010), particularly for enterprises in southern Australia (Besier, 1996; Love and Coles, 2002; Besier and Love, 2003; Woodgate and Besier, 2010). With sheep enterprises running higher stocking rates and the increased use of fertilisers to achieve faster pasture growth rates, strongylid egg contamination onto paddocks has increased. The existence of a mentality, which involves the common practices of both regular whole-flock anthelmintic treatment, followed by the movement of sheep to a clean or rested pasture, has advanced the contamination of pastures with resistant larvae, from those resistant worms which have survived an anthelmintic treatment (Besier, 2007; Stafford et al., 2009; Dobson et al., 2011).
2.4.5.1 Anthelmintic treatment and resistance

At present there are only four groups of broad spectrum anthelmintics available for the treatment of strongylid worms in grazing sheep to minimise production losses (Table 2.2). These include Group 1; the benzimidazoles (BZ) (white fluid), Group 2; imidazothiazoles e.g. levamisole (LV) and Tetrahydropyrimidines (pyrantel/morantel, colourless fluid), Group 3: the macrocyclic lactones (ML) e.g. avermectins and milbemycins, and Group 4: amino-acetonitrile derivatives (monepantel) (Coles et al., 2006; Kaminsky et al., 2008a)

Since anthelmintic treatments were incorporated into sheep enterprise management programs in the 1960s, resistance has been found in the three major strongylid species which infect sheep (McKellar and Jackson, 2004; Waller, 2006), including Trichostrongylus spp. (Gopal et al., 2001), T. circumcincta and H. contortus (Sangster and Dobson, 2002; Kaplan, 2004; Stear et al., 2009). Resistance is present when there is a greater frequency of individuals within a worm population able to tolerate doses of anthelmintics compared to a normal unexposed population of the same species (Prichard et al., 1980). Resistance of strongylid worms to anthelmintics is common in WA, with T. circumcincta presenting the greatest resistance challenge (Palmer et al., 2001; Besier and Love, 2003; Suter et al., 2005). Besier (1996), reported that lambs treated with an anthelmintic which was 65% effective, suffered three times more disease infection (mortalities and diarrhoea) than lambs administered with a fully effective anthelmintic. Furthermore lambs treated with an 85% effective anthelmintic, suffered twice the degree of disease caused by strongylids, when a similar comparison was made.
Table 2.2: Classes of broad spectrum anthelmintics and resistance prevalence in Australian sheep (Adapted from Coles et al. 2006; Kaminsky et al. 2008a; Hosking et al. 2009).

<table>
<thead>
<tr>
<th>Class</th>
<th>Examples</th>
<th>Estimated prevalence of resistance</th>
<th>Major mode of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzimidazoles (BZ)</td>
<td>Albendazole, Fenbendazole</td>
<td>99 percent of WA properties</td>
<td>Disruption/Inhibition of Microtubules</td>
</tr>
<tr>
<td>Imidazothiazoles (LZ)</td>
<td>Levamisole, Tetramisole</td>
<td>99 percent of WA properties</td>
<td>Nicotinic acetylcholine receptor agonists</td>
</tr>
<tr>
<td>Tetrahydropyrimidines</td>
<td>Morantel, Pyrantel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BZ/LZ combination anthelmintics</td>
<td>Scanda, Nucombe</td>
<td>80 percent of WA properties</td>
<td>Combination of the above modes of action</td>
</tr>
<tr>
<td>Macrocyclic lactones (ML)</td>
<td>Ivermectin, Doramectin</td>
<td>60 percent of WA properties</td>
<td>Glutamate-gated chloride channel agonists</td>
</tr>
<tr>
<td>Avermectins</td>
<td>Abamecin, Moxidectin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milbemycins</td>
<td>Doramectin, Milbemycin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organophosphates (OP)</td>
<td>Naphthalophos</td>
<td>Only one recorded case in Australia</td>
<td>Interfere with worm nervous system</td>
</tr>
<tr>
<td>Closantel</td>
<td>Cosal, Razar</td>
<td>Resistance in NSW and Queensland in H. contortus</td>
<td>Perturbation of mitochondrial membranes</td>
</tr>
<tr>
<td>Amino-acetonitrile derivatives</td>
<td>Monepantel</td>
<td>None</td>
<td>Acts on the nervous system worms, targeting HCo-MPTL-1 receptors only present on worms</td>
</tr>
</tbody>
</table>

Strategies to better manage anthelmintic resistance together with improved strongylid control programs both remain commercially compelling issues to those who either work or have an interest in the sheep industry. Multiple-anthemintic resistance has been reported from major sheep producing countries, including Australia, South America, New Zealand and South Africa (Besier and Love, 2003; Kaplan, 2004; Sutherland et al., 2010; van Wyk and Reynecke, 2011). With the reduced effectiveness of older anthelmintic groups, overuse of newer, higher potency anthelmintics have contributed to the development of multiple-anthelmintic group resistance in strongyloid worm populations (Gopal et al., 2001; Palmer et al., 2001; Sargison et al., 2010). Multiple-anthelmintic group resistance is present in Western Australian sheep enterprises, as shown predominantly by...
T. circumcincta and ML resistance present in ~84% of faecal egg count reduction tests from 2003 to 2005 (Besier, 2006).

However, with the release of Monepantel in 2010 (Zolvix®, Novartis Australia; the first amino-acetonitrile derivative anthelmintic), a new anthelmintic available to both Australian and United Kingdom sheep production, it is possible that it too will be overused thereby reducing it’s longevity. Monepantel acts on the nervous system of strongylid worms, targeting HCo-MPTL-1 receptors only present on the strongylid worms (Kaminsky et al., 2008a; Kaminsky et al., 2008b; Hosking et al., 2009; Kaminsky et al., 2009). There is incredible potential for this new anthelmintic as it has demonstrated 99% effectiveness against susceptible and resistant strongylid species strains, although strategic use of this new anthelmintic must be promoted to reduce the early onset of worm resistance (Kaminsky et al., 2008a; Kaminsky et al., 2008b; Hosking et al., 2009; Sager et al., 2009; Dobson et al., 2011).

2.4.5.2 Strongylid worm control programs

The common practice of administering an anthelmintic to sheep during summer when the conditions for worm survival on pasture and transmission are poor, is referred to as “summer drenching” (Besier and Love, 2003; Woodgate and Besier, 2005; Besier, 2009; Woodgate and Besier, 2010). Although summer administration of anthelmintics has provided effective strongylid worm control for sheep enterprises in southern WA, it also enables the survival of resistant worms, in preference to susceptible worms. Continuous summer anthelmintic use over time has contributed to an increase in anthelmintic resistance on sheep properties (Besier, 2009; Woodgate and Besier, 2010). Worms in ‘refugia’ are those worms not exposed to a chemical anthelmintic. There is evidence in WA
sheep enterprises that managers who leave a small percentage of sheep untreated (i.e. maintain populations of worms in refugia), reduce the emphasis of selection for anthelmintic resistant worms, as both resistant and susceptible larvae eggs will be passed in the faeces onto pasture (Love, 2007; Besier, 2009; Woodgate and Besier, 2010).

Non-chemical strategies to maintain control of strongylid worms are recommended and these include the adoption of a strategic clean grazing system; which involves rotation of sheep, cattle and crop paddocks on an annual basis (Mitchell and Fitzsimons, 1983; Jackson et al., 2009), as well as maintaining a small proportion of sheep untreated (refugia) at the time of a summer anthelmintic administration (Love, 2007; Woodgate and Besier, 2010). Alternative approaches include the management of free living strongylid parasite populations by use of predatory fungi, such as Duddingtonia flagrans (Waller and Faedo, 1996; Larsen, 1999) and bioactive forage grazing, particularly that of tannin rich forages: Cichorium intybus (chicory), Lotus corniculatus (birdsfoot trefoil) and Onobrychis viciifolia (sainfoin) (Heckendorn et al., 2007; Athanasiadou et al., 2008; Max, 2010; Valderrábano et al., 2010).

2.4.6 DIAGNOSIS

The definitive diagnosis of helminthosis typically involves the investigation of faecal samples for the presence of strongylidea eggs and/or post-mortem examination involving a total worm count. The McMaster worm egg count (WEC) flotation (Whitlock, 1948; Lyndal-Murphy, 1993) is a widely utilised microscopic technique for the diagnosis and quantification of strongylid worm infections. The McMaster technique has been shown to be a useful guide to patent worm burdens (Kingsbury, 1965; McKenna, 1981), but cannot distinguish all of the different strongylid species that contribute to an individual WEC.
without the use of larval culture and differentiation. Larval culture differentiations are time consuming, require skilled laboratory staff, depend on strictly controlled culture conditions (temperature and humidity) to prevent a species culture with bias and require relatively large volumes of faecal material (Dobson et al., 1992; McKenna, 1998; Tyrrell et al., 2002). As a result they are typically only performed on pooled (multiple) samples of faeces (Dobson et al., 1992). Strongylid L₃ on pastures are detected by performing pasture larval counts, to determine the number of different strongylid larvae species present. The method is capable of detecting all larval stages of strongylid species, however pasture larval counts are time consuming (requiring extensive washing, soaking, filtration and centrifugation techniques to separate larvae from pasture and debris), labour intensive and expensive to perform (Martin et al., 1990; Fontenot et al., 2003).

Another diagnostic method utilised for species-specific identification of strongylid infections is the lectin binding assay that differentiates worm eggs using genus-specific carbohydrates found on the surface of eggs (Palmer and McCombe, 1996; Greenhalgh et al., 1999; Colditz et al., 2002; Jurasek et al., 2010). This method has proved to be useful for the detection of H. contortus and Trichostrongylus spp. infections in sheep, with strong correlations found to those observed in larval culture results and no evidence of an effect dependent upon the stage of nematode egg development (Palmer and McCombe, 1996; Colditz et al., 2002). At present, lectins specific for T. circumcincta, Oesophagostomum spp. or Chabertia ovina, have not been identified.

More recently, a faecal occult blood assay utilising a commercial “Haemonchus Dipstick Test” has been developed. This method has a short processing time (~30 mins) and another advantage is that samples can be tested on-farm, rather than in a laboratory.
However, the test is not quantitative and both false positive and negative test results have been reported (Colditz and Le Jambre, 2008).

Recently, a molecular method for the species-specific diagnosis of strongyloid infections was developed by Bott et al., (2009). A polymerase chain reaction (PCR) and quantitative real-time polymerase chain reaction (qPCR) were successfully used by Bott et al., (2009) to identify faecal samples positive for strongylidea worms. In addition, this test is able to confirm the presence of individual strongyloid species in genomic DNA extracted from column purified worm eggs and has the potential to quantify infection levels. The PCR primers target species-specific genetic markers at the internal transcribed space (ITS-2) of nuclear ribosomal DNA (rDNA) (Bott et al., 2009; Learmount et al., 2009). Although this technique is useful, it is currently not quantitative and has only been tested on DNA extracted from eggs purified from faeces by sodium nitrate flotation and column-purification (Bott et al., 2009; Hunt, 2011; Roeber et al., 2011). There are no comparisons which examine the level of agreement between the McMaster WEC flotation method and PCR diagnostic assays, for the detection of patent strongyloid infections using genomic DNA extracted directly from faeces. However comparisons between McMaster WEC and PCR have been conducted, with PCR assays screening genomic DNA extracted from worm eggs following column-purification from faecal material (Hunt, 2011; Roeber et al., 2011).

The most accurate test to definitely ascertain and quantify a strongyloid worm infection is by post-mortem examination, whereby a total worm count from the gastrointestinal tract of a sheep is conducted. This is the most conclusive test, although it obviously requires the sacrifice of chosen sheep.
2.5 CRYPTOSPORIDIUM INFECTIONS

The protozoan parasites reported to infect the gastrointestinal tract of vertebrates include *Cryptosporidium* spp., *Giardia* spp. and *Eimeria* spp. and all of these protozoa have been detected in sheep in WA (Yang et al., 2009). *Cryptosporidium* is a widely distributed ubiquitous, protozoan parasite of vertebrates and has attracted significant interest as a result of (1) several serious waterborne outbreaks due to prolonged oocyst survival in the environment and it's resistance to disinfectants, (2) the risk of infection for immune-compromised patients and (3) their zoonotic potential and wide host range. *Cryptosporidium* have been perceived to have negative economic impacts on livestock productivity (Plutzer and Karanis, 2009), although there has only been minor findings in feedlot cattle and sheep (Ralston et al., 2003; Aloisio et al., 2006; Giadinis et al., 2007). It is the most common enteric parasite for both humans and domestic animals, and also can be isolated from wildlife (Fayer et al., 2000a; Power et al., 2003; Appelbee et al., 2005; Thompson et al., 2005; Smith and Nichols, 2010; Xiao, 2010). Currently, the genus *Cryptosporidium* encompasses 24 species and over 45 genotypes, with *Cryptosporidium hominis* and *Cryptosporidium parvum* globally recognized as the most important species infecting humans (Xiao and Fayer, 2008; Plutzer and Karanis, 2009; Fayer, 2010; Smith and Nichols, 2010; Xiao, 2010).

Infections of *Cryptosporidium* in cattle, sheep, pigs and horses have been reported in countries worldwide (Chalmers et al., 2002; Delafosse et al., 2006; Santin et al., 2007; Fayer et al., 2010b; Castro-Hermida et al., 2011; Fiuza et al., 2011).

Ovine cryptosporidiosis, was first described in diarrheic lambs in Australia by Barker and Carbonell (1974) and has subsequently been reported in 12 other countries (Fayer and
Santin-Duran, 2009), with the highest prevalence found most often in lambs near the end of their neonatal period (less than 1 month of age) (Majewska et al., 2000; O'Handley and Olson, 2006; Santin et al., 2007; Yang et al., 2009; Robertson et al., 2010; Wang et al., 2010b).

The infective stages of both Cryptosporidium and Giardia, are encysted when released in faeces and are capable of prolonged survival in a variety of different environments (Robertson et al., 1992; Fayer et al., 1996; Olson et al., 1999; Carey et al., 2004). Once in the environment, re-infection is achieved by consumption of contaminated feed or water, or by direct host to host contact. Different species of flies have been reported to be capable of carrying and enhancing the transmission of both Cryptosporidium and Giardia between livestock, particularly those intensively managed within confined barns, stalls, feedlots or during blowfly strike outbreaks (Graczyk et al., 1999; Graczyk et al., 2005; Fetene et al., 2011).

2.5.1 TAXONOMY

The taxonomic status of Cryptosporidium is undergoing rapid change as further research is conducted. The taxonomic determination can be described as follows: Cryptosporidium spp. belong to the phylum Apicomplexa (=Sporozoa), whose members possess an apical complex; class Sporozoae, whose members reproduce using asexual and sexual cycles; subclass Coccidia, the life cycle of which involves merogony, gametogony and sporogony; order Eucoccidiida (=Eucoccidiorida), in which schizogony occurs; suborder Eimeriina (Eimeriorina), in which micro- and macrogamy develop; and family Cryptosporidiidae, whose members have four naked sporozoites within their oocysts (Plutzer and Karanis, 2009; Fayer, 2010). This original classification resulted from
similarities found for this parasite, when compared with the coccidian life cycle (Hijjawi et al., 2002; Hijjawi, 2010). However molecular and biological studies indicate that Cryptosporidium have a closer affinity with the gregarines rather than with coccidia (Bull et al., 1998; Carreno et al., 1999; Hijjawi et al., 2002; Leander et al., 2003; Rosales et al., 2005; Zhang et al., 2009; Hijjawi et al., 2010).

Until recently, most studies of cryptosporidiosis in sheep were conducted using microscopy only and assumed that sheep were infected with C. parvum only. However, with the application of molecular epidemiology tools, discoveries of a wide variety of species/genotypes have been identified in sheep faeces: C. parvum, C. suis, C. andersoni, C. hominis, C. bovis, C. xiaoi (formerly C. bovis-like genotype), C. ubiquitum, pig genotype II and C. fayeri (McLauchlin et al., 2000; Chalmers et al., 2002; Ryan et al., 2005; Pritchard et al., 2007; Santin et al., 2007; Elwin and Chalmers, 2008; Mueller-Doblies et al., 2008; Fayer and Santin-Duran, 2009; Yang et al., 2009; Robertson et al., 2010; Wang et al., 2010b; Fiuza et al., 2011).

Cryptosporidium xiaoi and C. bovis are genetically very similar, with identification of C. xiaoi based solely on the similarly aligned nucleotide sequences of the actin gene compared with C. bovis (Fayer and Santin-Duran, 2009). This was confirmed as a new species of Cryptosporidium in sheep by Fayer and Santín (2009), previously known as Cryptosporidium bovis-like genotype.

Recently, Fayer et al. (2010b) also reported Cryptosporidium ubiquitum as a new species. Cryptosporidium ubiquitum has been found in both animals and humans and has been confirmed as a new species by comparing nucleotide sequences and their genetic differences at three independent loci, along with photomicrograph microscope morphology.
(Fayer et al., 2010b). This species, which was previously referred to as a cervine genotype, Cryptosporidium cervid, W4 and genotype 3 has it’s status clarified in this publication as a well documented Cryptosporidium species found in sheep worldwide (Ryan et al., 2005; Elwin and Chalmers, 2008; Yang et al., 2009; Robertson et al., 2010; Wang et al., 2010b; Fiuza et al., 2011). Cryptosporidium ubiquitum has been reported in humans (Ong et al., 2002; Learmonth et al., 2004; Chalmers et al., 2009), the environment (including human water sources) (Bodley-Tickell et al., 2002; Feng et al., 2007; McCarthy et al., 2008; Nichols et al., 2010; Ng et al., 2011b) and a wide variety of native and feral animals (Power et al., 2003; Power et al., 2004; Castro-Hermida et al., 2011). However, there is currently no evidence of it’s detection in any human cases of cryptosporidiosis in Western Australia (Ng et al., 2010a; 2010b).

2.5.2 LIFE CYCLE AND TRANSMISSION

Cryptosporidium spp. have a complex monoxenous life cycle in which all stages of development (both asexual and sexual) occur within the host (Figure 2.8). This life cycle is direct and the infective stages of the parasite are encysted as oocysts. When these are released in the faeces of infected livestock, they are immediately infectious (Kirkpatrick, 1987). In favourable cool, damp environmental conditions and also in water, the thick walled oocysts remain infectious for between 3–6 months (Robertson et al., 1992; Fayer et al., 1996; Olson et al., 1999; Carey et al., 2004). Their survival in submerged semi-solid cow faeces, at ambient temperatures was reported for periods of 5–6 months (Robertson et al., 1992; Olson et al., 1999). Oocysts found in water bodies where the temperature is up to 23–25°C will survive for ~3 months (Fayer et al., 1996; Carey et al., 2004). The host ingests the sporulated oocysts (round ovoid structures typically measuring 4-6µm), from which the sporozoites are released by excystation, due to the warm aqueous solutions and...
gastric acids found in the gastrointestinal lumen (Reduker and Speer, 1985). After excystation, the endogenous phase of the life cycle begins, as sporozoites penetrate host cells and develop into trophozoites within parasitophorous vacuoles located on the villous brush border region of the mucosal epithelium (Current, 1990; Carey et al., 2004). Trophozoites undergo asexual division (by merogony) to form merozoites and after invading host cells they form either type I or type II meronts. Type II meronts commence the intracellular sexual phase of their Cryptosporidium life cycle (Current and Reese, 1986; Current, 1990; Carey et al., 2004; Thompson et al., 2005). The majority (~80%) of zygotes formed from intracellular fertilisation develop into thick walled resistant oocysts, which undergo sporogony to form sporulated oocysts that are released in faeces. A small percentage (~20%) of zygotes form thin walled oocysts, which are important in the autoinfection process of the host through the rupturing of thin walled oocysts (Figure 2.8). This allows autoinfection of the host without further exposure to thick walled oocysts from the environment (Current, 1990; Carey et al., 2004; Thompson et al., 2005).
CHAPTER 2: LITERATURE REVIEW

2.5.3 CLINICAL SIGNS, AND PATHOLOGY

*Cryptosporidium* infections are commonly found in the micro-villous border in the gastrointestinal tract of vertebrate hosts (Current and Blagburn, 1990; Fayer *et al*., 2000a). Such infections are usually associated with clinical signs of diarrhoea and gastro-enteritis in infected animals, (Olson *et al*., 1997; Causapé *et al*., 2002; Wilkes *et al*., 2009), but also can infect the respiratory tract if inhaled. Infection can often be subclinical (Xiao *et al*., 1993; Aloisio *et al*., 2006). If clinical signs manifest, they are commonly observed in ï or pregnant animals (de Graaf *et al*., 1999b; Ortega-Mora *et al*., 1999; Castro-Hermida *et al*., 2001; O’Handley and Olson, 2006; Giadinis *et al*., 2007) or animals kept in confined
situations; such examples include livestock feedlots or piggeries. Livestock confined to areas where overcrowding, stress and nutritional deficiency are encountered, promote both transmission and exacerbation of Cryptosporidium infections (Thompson et al., 2008).

Pathogenesis of Cryptosporidium infections is associated with the interaction between the parasite’s products and the host’s immunological and inflammatory responses (Thompson et al., 2008). Cryptosporidium infections are predominantly in the small intestine, causing enterocyte apoptosis, which is linked to the disruption of tight junction proteins (Koudela and Jirí, 1997; Chai et al., 1999; Chin et al., 2002), villous fusion and shortening, villous atrophy and inflammation of the small intestine (Koudela and Jirí, 1997). As a result of these pathogenic effects there is a reduction in the absorptive surface area, interference of nutrient transport, reduction in nutrient absorption and cell apoptosis in the gastrointestinal tract (Buret et al., 2003), resulting in the clinical signs associated with diarrhoea and weight loss (Olson et al., 1997; Thompson et al., 2005; Thompson et al., 2008). The pathogenesis of the disease is commonly complicated by the concurrent presence of bacterial (Salmonella), viral (rotavirus) and other internal parasites (strongylid worms and Giardia) (Fayer et al., 1998; de Graaf et al., 1999a; de Graaf et al., 1999b; O'Handley et al., 1999). It still remains unclear how the majority of the above pathophysiological changes occur, as infection is not always associated with the presence of clinical signs.

Cryptosporidium andersoni resides in the abomasum of ruminants, commonly infecting post-weaned and mature cattle, with often asymptomatic infections of this species reported (Anderson, 1998; Fayer et al., 2005). Cryptosporidium andersoni has been reported to cause stretching of the peptic and pyloric glands, hypertrophy of gastric
mucosa and thinning of the epithelial lining (Anderson, 1987; Olson et al., 2004). Yang et al. (2009) reported one lamb positive for *C. andersoni* in a recent WA study, although this abomasal *Cryptosporidium* species has not been well documented in sheep and as a result sheep are perceived to act as a minor host, with no clinical signs of infections have been reported (Anderson, 1998; Smith and Nichols, 2010).

### 2.5.4 PRODUCTION LOSS

*Cryptosporidium* infections in sheep and lambs have been extensively studied, nearly entirely for their molecular epidemiological significance regarding zoonotic transmission potential and the contamination risks posed for human water sources. In contrast there is no literature indicating the impact of infections on reduced growth rates, lower live weights and reduced carcass yields for lambs extensively grazed. Giadinis et al., (2007) examined the effect that treatment against *Cryptosporidium* with halofuginone lactate had on 1–12 day old lamb growth rates when one flock was confirmed with cryptosporidiosis. The lambs from this latter flock were split into two groups, one group receiving treatment with halofuginone lactate and the other group remaining untreated. There was no significant change in growth rate between the treated and untreated group, although oocyst output was significantly reduced in the treated group. Giadinis et al., (2007) suggested that *Cryptosporidium* infections have little significance on lamb live weight and growth rate, with the halofuginone lactate treatment only beneficial in reducing both the oocyst output and the transmission risk of the parasite to other flock members.

In another study by Castro-Hermida et al., (2004), when 6 day old goat kids were challenged by oral infection with *C. parvum* oocysts, one group being treated with α-cyclodextrin while the the other group remained untreated, there was no significant
difference in growth rates between the two groups. Similarly, this was also observed in an earlier study involving neonatal lambs with cryptosporidiosis, whereby one group was treated with β-cyclodextrin and compared to an untreated group (Castro-Hermida et al., 2001). In Spain, the presence of C. parvum was significantly associated with diarrhoea in all age groups examined (1 day to 1 year) (n=868) (Causapé et al., 2002).

In cattle, 60 weaned Charolais crossbred steers were introduced to pens and faecal samples collected at four different occasions over 175 – 257 days, with all animals monitored for dry matter intake, average daily liveweight gain and feed efficiency (Ralston et al., 2003). In comparing Cryptosporidium infected and non-infected steers, there was no significant difference in the average daily liveweight gain and feed efficiency. However, steers with C. andersoni infections had an overall significantly reduced dry matter intake when compared to uninfected steers (Ralston et al., 2003).

2.5.5 DIAGNOSIS

Microscopic examination of faecal samples is the most common laboratory approach to confirm whether a sample is positive for Cryptosporidium (Thompson et al., 2008). However, the resemblance of oocysts to background faecal debris can make identification difficult for the inexperienced microscopist, with previous microscopic studies reported to have less diagnostic sensitivity compared to PCR assays (Morgan-Ryan et al., 1998; Fayer et al., 2000a; Li et al., 2010). To enhance oocyst visibility, there are multiple staining techniques, although their sensitivity and specificity often leads to variable laboratory results (Elliot et al., 1999). Malachite green staining for detecting Cryptosporidium oocysts employs a negative staining technique, which has provided good
results when compared to other staining methods, however, the method still requires a skilled and experienced microscopist (Elliot et al., 1999).

Other diagnostic tools are available to improve the sensitivity of conventional staining methods. These include enzyme-linked immunosorbent assay (ELISA) and immunofluorescent assay based methods. Drawbacks to these methods include the high cost, cross reaction with non-target organisms, an inability to differentiate between species and lack of specificity (Fayer et al., 2000a; Wang et al., 2004; Thompson et al., 2008) (Wang et al. 2004; Thompson et al. 2008).

2.5.5.1 Nucleic acid detection based on PCR and qPCR

The development of nested PCR techniques offers high levels of sensitivity and specificity (Morgan-Ryan and Thompson, 1998), when testing faecal samples for Cryptosporidium spp. and further allows for species identification. Multiple nucleic acid regions of Cryptosporidium, particularly the 18S rRNA (Xiao et al., 1999), the actin gene (Sulaiman et al., 2002) and 60kDa glycoprotein gene (gp60) (Strong et al., 2000) are beneficial for detecting and typing a variety of Cryptosporidium species/genotypes. Furthermore, QPCR has been effectively used to quantify Cryptosporidium infections in lambs (Yang et al., 2009), with increased sensitivity and specificity, when compared to normal PCR (Santin et al., 2007; Mueller-Doblies et al., 2008; Yang et al., 2009).

2.5.6 TREATMENT AND CONTROL

The need to treat Cryptosporidium infections in ruminants is debatable (O'Handley and Olson, 2006; Thompson et al., 2008) for two main reasons. Not only are the chemotherapeutic agents expensive, but there is also a high risk of re-infection when treated animals are released back to either the extensive grazing of pastures or intensive
housed/barn environments. Some chemotherapeutic agents have shown promise in sheep, goats and cattle suffering from cryptosporidiosis, with examples including paromomycin (Griffiths et al., 1998; Viu et al., 2000; Rossignol, 2010), halfuginone lactate (Giadinis et al., 2007; Giadinis et al., 2008; De Waele et al., 2010), nitazoxanide (Plutzer and Karanis, 2009; Schnyder et al., 2009; Rossignol, 2010) (Schnyder et al. 2009; Rossignol 2010), cyclodextrin (Castro-Hermida et al., 2001; Castro-Hermida et al., 2004) and lasalocid (Castro-Hermida et al., 2000). The practicality of these chemotherapeutic agents is questionable, as they are yet to be proven cost-effective for livestock enterprises (Olson, 2000). Halfuginone lactate (Halocur®, Intervet Schering-Plough Animal Health) has been used to treat goat kids for diarrhoea, Cryptosporidium oocyst output and to reduce the duration of oocyst shedding. It has been reported to have a significant effect (P<0.05) in reducing the incidence of diarrhoea and in minimising case fatality (Giadinis et al., 2008). However treatment of 100µg/kg halfuginone lactate/day for 7 days, would be difficult for large scale livestock enterprises and is perceived as being unwarranted by many livestock producers. This is due to the high risk of re-infection when treated animals are re-introduced into their pre-treatment environment (paddock, pen or barn) following treatment and in addition regular chemotherapeutic follow up treatment would be necessary to limit infections (O'Handley and Olson, 2006; Thompson et al., 2008). Halfuginone lactate is registered in Europe, New Zealand and the USA as a chemotherapeutic treatment for cryptosporidiosis in cattle, reducing both the incidence and severity of diarrhoea (Olson et al., 2004). However the treatment is not as effective in reducing oocyst shedding, an outcome that was also observed for the chemotherapeutic agent nitazoxanide (Alinia® Ronmark Laboratories) (Giadinis et al., 2008; Schnyder et al., 2009). Although nitazoxanide chemotherapy appears promising after recently being licensed in the USA for
treating cryptosporidiosis and giardiasis (Rossignol, 2010). Schnyder et al., (2009) found nitazoxanide did not produce an expected ant-cryptosporidial effects in treated versus untreated neonatal calves.

2.5.6.1 New drug targets and new treatments

The epidermal growth factor membrane receptors of Cryptosporidium spp. have been identified as targets of the naturally occurring genistein and a new family of isoflavone derivatives showing in vitro and in vivo activity against Cryptosporidium spp. (Stachulski et al., 2006). A distinctive C. parvum enzyme called nonspecific polyphosphoryl pyrophosphate synthase has also been identified as a target for nitrogen containing bisphosphates, although the efficacy against other Cryptosporidium species is uncertain (Artz et al., 2008).

2.6 GIARDIA INFECTIONS

Giardia duodenalis (syn. Giardia intestinalis and Giardia lamblia) is a zoonotic flagellated protozoan parasite that causes giardiasis in humans, pets, livestock and wildlife. (Taylor et al., 1993; Xiao et al., 1994; Olson et al., 1995; Olson et al., 1997; Geurden et al., 2010b; Feng and Xiao, 2011). Diarrhoea associated with Giardia spp. has been reported in young cattle, sheep and horses (Xiao et al., 1993; Olson et al., 1995; O’Handley et al., 1999; O’Handley and Olson, 2006; Muhid et al., 2011). Lambs that are infected with Giardia spp. usually display clinical signs of diarrhoea, reduced weight gains (Taylor et al., 1993; Olson et al., 1995; Aloisio et al., 2006), an impaired feed efficiency (Olson et al., 1995) and reduced feed intake (Ralston et al., 2003). As with Cryptosporidium, sheep commonly become infected with Giardia through inadvertently ingesting pasture or supplementary feed contaminated with cysts (Taylor et al., 1993; Olson et al., 1997; O’Handley and Olson, 2006). It is also common for livestock to become infected with
*Giardia* via the consumption of contaminated water (Olson *et al*., 2004; O’Handley and Olson, 2006; Thompson *et al*., 2008).

### 2.6.1 TAXONOMY

There are six established species of *Giardia* recognised on the basis of host infection patterns and morphological characteristics (Feng and Xiao, 2011). Of these, *G. duodenalis* is known to infect mammals and vertebrates, with the genotype assemblages of *G. duodenalis* outlined in Table 2.3. Presently genotypes are described as assemblages, however a revision of the taxonomy has been proposed (Ortega-Pierres *et al*., 2009; Feng and Xiao, 2011). The present taxonomic position of the genus *Giardia* is within the family of Hexamitidae, order Diplomonadida, class Zoomastigophora, subphylum Mastigophora of the Phylum Sarcomastigophora in the protozoan kingdom (Feng and Xiao, 2011).
Table 2.3: Genetic genotypes of *G. duodenalis*

<table>
<thead>
<tr>
<th>Assemblage (genotype grouping)</th>
<th>Host Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assemblage A</td>
<td>Human, primates, dogs, cats, domestic ruminants, rodents, horses, marsupials, pigs and other mammals</td>
</tr>
<tr>
<td>Assemblage B</td>
<td>Humans, primates, cattle, dogs, horses, rabbits and beavers and other mammals</td>
</tr>
<tr>
<td>Assemblage C/D</td>
<td>Dogs, both domestic and wild</td>
</tr>
<tr>
<td>Assemblage E</td>
<td>Domestic ruminants (cattle, sheep and goats) and pigs</td>
</tr>
<tr>
<td>Assemblage F</td>
<td>Cats</td>
</tr>
<tr>
<td>Assemblage G</td>
<td>Rodents; mice and rats</td>
</tr>
<tr>
<td>Assemblage H</td>
<td>Seals</td>
</tr>
</tbody>
</table>

Adapted from (Thompson and Monis, 2004; Lasek-Nesselquist et al., 2010; Feng and Xiao, 2011).

Infections with *Giardia* in sheep have been reported worldwide and sheep have been implicated as a potential reservoir for zoonotic transmission to humans (Buret *et al.*, 1990a; Zajac, 1990; Xiao, 1994; Olson *et al.*, 1995; Olson *et al.*, 1997; Ryan *et al.*, 2005; Gómez-Muñoz *et al.*, 2009; Yang *et al.*, 2009; Lebbad *et al.*, 2010; Nolan *et al.*, 2010). Recent studies have provided detail for the prevalence of different *Giardia* assemblages in livestock, with a primary focus on the prevalence of two different assemblages for *G. duodenalis*; assemblage A and assemblage E (Thompson, 2004; Thompson and Monis, 2004; Santin *et al.*, 2009; Yang *et al.*, 2009; Nolan *et al.*, 2010). More recent research in Maryland, USA (Santin *et al.*, 2007), the south-west of WA (Yang *et al.*, 2009), Victoria, Australia (Nolan *et al.*, 2010) and Van Province, eastern Turkey (Ozdal *et al.*, 2009) respectively, have identified both assemblages A and E in sheep. Yang *et al.*, (2009) identified sheep in Western Australia with assemblage A, assemblage E and mixed infections of A and E. There have been reports of assemblage B infecting lambs in Italy.
(Aloisio et al., 2006), Sweden (Lebbad et al., 2010), Norway (Robertson et al., 2010) and Spain (Castro-Hermida et al., 2007).

2.6.2 LIFE CYCLE AND TRANSMISSION

Unlike Cryptosporidium, the life cycle of Giardia is simple and involves just two major stages; a cyst and a trophozoite (Figure 2.9). Giardia cysts are environmentally resistant and capable of survival in ambient environments for many months (Figure 2.11) (Erickson and Ortega, 2006; Huang and White, 2006; Pierce and Huston, 2009). The dormant, infectious cysts (ovoid structure 7-10µm wide and 8-12 µm in length) are transferred usually in faecal material, ingestion of contaminated feed or water, or by direct animal to animal contact (Thompson et al., 2008; Geurden et al., 2010b; Feng and Xiao, 2011). Once ingested by a suitable host, the low pH associated with gastric acids and warm, aqueous environment triggers excystation, releasing trophozoites from the broken down cysts into the duodenum of the small intestine (Figure 2.9). Each released trophozoite undergoes cytodifferentiation and division giving rise to two new trophozoites (Figure 2.9 stage a). These trophozoites use their flagella to migrate along the small intestine, where they infect the microvilli of the small intestine. Attachment of some trophozoites to the small intestinal microvilli is assisted by a unique ventral sucking disc, allowing the parasite to insert itself between the microvilli of the small intestine (Figure 2.9 stage b) (Binz, 1996; Thompson et al., 2008; Pierce and Huston, 2009; Feng and Xiao, 2011).
Figure 2.9: Diagrammatic representation of the life cycle of Giardia spp. of an infected mammalian host. Adapted from Binz (1996) and Hobbs et al., (2007).

Trophozoites (Figures 2.9 and 2.10) multiply by binary fission and colonise the duodenum and the jejunum of the small intestine. The majority of trophozoites avoid being washed further down the gastrointestinal tract because of their strong attachment to the small intestinal wall via their ventral sucking disc. Some trophozoites get flushed through into the large intestine by (i) regular sloughing of intestinal epithelium, (ii) loss of the ability to maintain a strong attachment as enabled by a ventral sucking disc and (iii) newly divided trophozoites cannot find a microvillus surface to which they can attach (common for heavy infections in the small intestine) (Binz, 1996; Thompson et al., 2008; Pierce and Huston, 2009; Feng and Xiao, 2011). Once in the ileum and the upper large intestine, the presence of bile leads to an increased pH, which triggers encystation; the release of cysts by a trophozoite. The cysts lack any attachment mechanisms and therefore are excreted in the faeces, where they can remain infective for months under appropriate temperature and moisture conditions (Figure 2.9, stage c.) (Erickson and Ortega, 2006; Huang and White, 2006; Pierce and Huston, 2009).
2.6.3 CLINICAL SIGNS AND PATHOLOGY

Clinical signs include osmotic (absorptive) diarrhoea due to disruption of the epithelial brush border in the small intestine. Studies involving human epithelial cell lines (Buret et al., 1990b), laboratory animals (Buret et al., 2002; Scott et al., 2002) and calves (O'Handley et al., 2001), reveal that giardiasis essentially leads to microvillus alterations, which include a decreased crypt to villous ratio and brush border enzyme deficiencies. In addition the pathophysiological responses to giardiasis are not only a consequence of trophozoite and epithelium interaction, but also mediated by the host’s immune response (Scott et al., 2000).

Similar to cryptosporidiosis, Giardia infections are more commonly implicated as a cause of diarrhoea in lambs rather than adult sheep, particularly for livestock confined to a reduced area, where overcrowding, stress and direct contact, all increase the risk of such infections spreading (O'Handley and Olson, 2006; Thompson et al., 2008). The link between infection and diarrhoea is open to question, as diarrhoea has not been consistently associated with giardiasis (Taylor et al., 1993; Radostits et al., 1994; Olson et al., 1997; Besier et al., 2004). Differences in clinical severity have been attributed to differences in pathogenicity of Giardia genotype assemblages, parasite burdens, gastrointestinal microflora and variations in host immunity (Buret et al., 1992; Olson et al., 1995; Geurden et al., 2010b).

Although these infections are prevalent in ruminant livestock, they often are asymptomatic, with infections either acute (lasting several days) or chronic (lasting several months characterised by yellow mucous in faeces) (Taylor et al., 1993; Xiao, 1994; Xiao et al., 1994; Olson et al., 1995; Yanke et al., 1998). Furthermore, studies have reported lambs
that are unable to clear naturally acquired protozoan infections or which were repeatedly re-infected from their environment or other flock members (Taylor et al., 1993).

Figure 2.10: Left – An electronic microscope image of the fine structure of a Giardia cyst in vivo, Right – The ventral surface of a Giardia trophozoite (Binz, 1996).

The pathophysiological changes associated with a Giardia infection include; shortening of and damage to microvilli, reduced disaccharide breakdown, loss of the epithelial barrier and alteration of tight junctions structure (Buret et al., 1990b; Buret et al., 1990c; Buret et al., 2002; Buret, 2007). These changes are thought to be due to a combination of both the parasite by-products and the host’s immune factors, particularly that of CD8+ cells (O’Handley et al., 2001; Buret, 2007). The colonisation of the mucosal surface of the small intestine by Giardia trophozoites cause microvillus shortening and a reduced absorptive surface area. Enzyme activity is also reduced, resulting in impaired digestion and reduced absorption of glucose, water and sodium from the small intestine (Buret et al., 1990b; Buret et al., 1990c; Buret et al., 1992; O’Handley et al., 2001; Buret et al., 2002). Trophozoite toxins and T-cell activation decrease the enzyme activities of lipases, proteases and the disaccharidases; lactase, maltase and sucrose (Buret et al., 2002).
1990c; Scott et al., 2002). The diffuse microvillus shortening leads to an impaired water, electrolyte and nutrient intake, which in turn may often result in diarrhoea (Buret, 2007). Giardia infections potentially cause hyper-secretion of chloride ions (Troeger et al., 2007), in addition to impairing glucose, water and sodium absorption in the small intestine (Buret et al., 1990c; Buret et al., 2002). These pathophysiological changes are reported to cause malabsorption, maldigestion or both, in lambs experimentally infected with Giardia (Olson et al., 1995).

2.6.4 PRODUCTION LOSS

Livestock infected with Giardia display diarrhoea and in some cases reduced growth rates (Olson et al., 1995; O’Handley et al., 1999; Aloisio et al., 2006). Olson et al., (1995) examined 47 bottle fed, specific pathogen free lambs, 23 of which were surgically infected with Giardia. Infected lambs displayed abnormal, non-pelleted faeces, reduced growth rate, reduced carcase weight and impaired feed efficiency, when compared to the uninfected control group of 24 lambs. In an outbreak of giardiasis on an Italian sheep farm, Giardia infected lambs (30–90 days of age) displayed malabsorption, decreased growth rate and reduced feed efficiency (Aloisio et al., 2006). After they were treated with fenbendazole for 3 consecutive days, the infected animals recovered from their clinical signs (Aloisio et al., 2006). This conclusion was based on PCR analysis on only 2 specimens and it is possible other pathogens may have been present and responsible for the clinical disease. It is possible that different Giardia assemblages may play a role in weight loss and diarrhoea, as has been suggested by Read et al., (2002) in children with diarrhoea. However, Olson et al., (1995) did not perform any molecular analyses to determine the Giardia assemblage(s) surgically introduced into the barn raised lambs. Aloisio et al., (2006) reported Giardia assemblage B infections, which is rarely isolated from livestock, with only
a few reports of this assemblage being isolated from sheep worldwide (Aloisio et al., 2006; Castro-Hermida et al., 2007; Lebbad et al., 2010). Furthermore, although Aloisio et al., (2006) reported an increased proportion of lambs with diarrhoea and reduced growth rates, no statistical analyses were conducted to firstly compare if the difference in growth rates between infected and uninfected lambs was significant and secondly to determine if there was a significant rise in the number of lambs displaying abnormal faeces. No analyses or theories were proposed to explain how these Giardia infections caused significant economic loss in sheep enterprises, even though economic loss was described as one of study’s concluding findings. In addition, there was no reference to another variable, that reduced growth rates of infected lambs would have resulted in them taking longer to reach target weights for slaughter and therefore incurred extra feed costs for this sheep enterprise.

In cattle, a study by Ralston et al., (2003) on 60 Charolais crossbred steers found that there were no significant differences in the average daily liveweight gain (growth rate), dry matter intake and feed efficiency of Giardia infected and non-infected animals.

2.6.5 DIAGNOSIS

Light microscopy remains the most common method utilised to examine faecal samples for the presence of Giardia cysts, even though new diagnostic procedures such as immunofluorescence microscopy and PCR, are more effective although are associated with increased costs (Thompson et al., 2008; Geurden et al., 2010b; Feng and Xiao, 2011). As Giardia cyst excretion is intermittent and sporadic, multiple faecal samples need to be examined over a period of 4–5 days to ensure an accurate diagnosis (O’Handley et al., 1999; Thompson et al., 2008). Giardia cysts can be stained with malachite green and
Lugol’s iodine to help with identification, although a significant disadvantage of microscopy is that a skilled and experienced microscopist is required to detect cysts of this parasite (Geurden et al., 2004).

### 2.6.5.1 Nucleic acid detection based on PCR

Polymerase chain reaction assays are primarily used to differentiate *Giardia* assemblages for taxonomical and epidemiological research. The most common gene used for genotyping is the 18S rRNA (Read et al., 2004). Other loci useful for differentiating between *Giardia* assemblages are the glutamate dehydrogenase (gdh) gene (Read et al., 2004; Thompson et al., 2008; Geurden et al., 2009), β-giardin gene (Cacciò et al., 2002; Lalle et al., 2005; Cacciò and Ryan, 2008) and the triosephosphate isomerase (tpi) gene (Sulaiman et al., 2003). A disadvantage associated with PCR testing is that it suffers interference from DNA inhibitors, particularly if the DNA is extracted from animal faecal samples, as interference commonly occurs more so in livestock faecal samples than in human samples (Geurden et al., 2009; Geurden et al., 2010b).

### 2.6.6 TREATMENT AND CONTROL

The need for treatment in ruminants to control *Giardia* is debatable (Olson et al., 1997; O’Handley and Olson, 2006; Thompson et al., 2008; Geurden et al., 2010b), although a number of studies have evaluated the efficiency of different compounds and their effectiveness in calves. Nitazoxanide has been used in calves and companion animals for symptomatic improvement (St Jean et al., 1987; Xiao et al., 1993). Nitazoxanide has been shown *in vitro* to be a promising chemotherapeutic agent against *Giardia* (Cedillo-Rivera et al., 2002; Rossignol, 2010). Nitroimidazoles, paromomycin, nitazoxanide, and benzimidazole chemicals are currently considered the best group of
chemotherapeutic agents for the treatment of giardiasis in ruminants (O’Handley et al., 2000; O’Handley et al., 2001; Thompson et al., 2008; Rossignol, 2010). The benzimidazoles, commonly found in many strongylid nematode anthelmintics used in WA, require consecutive days of administration (3–5 days) for the reliable treatment of giardiasis. However this is not a sustainable option, with strongylid worm resistance already proving a major challenge for sheep enterprises in southern Australia. Nitroimidazoles are considered an alternative chemotherapeutic treatment for giardiasis, although here too there are concerns of resistance developing (Rossignol, 2010).

With anthelmintic resistance in worm populations an ever growing problem confronting sheep producers, particularly in WA, the broad spectrum amino-glycoside antibiotic; paromomycin, offers greater efficiency against protozoa parasites, compared to the benzimidazole anthelmintic. The paromomycin antibiotic binds to small sub unit rRNA and inhibits protein synthesis (Edlind et al., 1990; Harris et al., 2001), by interacting with ribosomal subunits resulting in the misreading of mitochondrial RNA (mRNA) codons. The antibiotic has been shown to reduce Giardia cyst excretion in experimentally infected calves for a period in excess of 2 weeks (Geurden et al., 2006), although it is poorly absorbed with ~60–70% efficacy rating against giardiasis (Grinberg et al., 2002; Minenoa and Avery, 2003; Rossignol, 2010).

2.6.6.1 New drug targets and new treatments

Protein disulfide isomerases have recently been reported as promising new chemotherapeutic agents effective against giardiasis (Müller et al., 2007; Müller et al., 2009; Rossignol, 2010; Nillius et al., 2011). Hence the isomerase products will be beneficial for new potential chemotherapeutic treatments such as genistein, isoflavone
derivatives and isomerases to have greater efficacy against both *Giardia* and *Cryptosporidium* (Rossignol, 2010).

### 2.6.6.2 Control Programs

Although the chemotherapeutic agents nitroimidazole, paromomycin, nitazoxanide, and benzimidazole are effective against *Giardia*, treated livestock re-excrete cysts 2–3 weeks post-treatment, due to re-infection from either their environment or other livestock (Thompson *et al.*, 2008; Geurden *et al.*, 2010a; Geurden *et al.*, 2010b). Since *Giardia* cysts can survive for 1–2 months in faeces or soil (Olson *et al.*, 1999; Erickson and Ortega, 2006), an effective treatment period of 3–5 days is too short and ineffective at preventing re-infection from a contaminated environment. With intensively managed livestock in feedlots or barns, correct disinfection practices and livestock management can help reduce the infection rate (Geurden *et al.*, 2010b). For extensively managed ruminants on pastures, livestock management including time of lambing, husbandry practices, paddock rotations, livestock water source and stocking rate all have the potential to influence the spread of both *Giardia* and *Cryptosporidium*. This presents an opportunity for the development of livestock control programs to minimise both the risk of transmission and also the impact of protozoan parasites (Geurden *et al.*, 2006).

### 2.7 PROTOZOA ZOONOTIC TRANSMISSION

Within the *Cryptosporidium* and *Giardia* genera there are multiple species/genotypes that can infect domesticated livestock, wildlife, pets and humans. These species and genotypes exhibit a wide range of biological diversity, each varying in their ability to infect single or multiple hosts. With an increased focus on public health and animal welfare, recent research has focused on identifying the protozoan
species/genotypes associated with infections in both humans and animals. For successful zoonotic transmission, the protozoan species/genotype must have zoonotic potential and be capable of transmission from animals to humans (Hunter and Thompson, 2005). *Cryptosporidium* and *Giardia* species/genotypes in domestic livestock are considered major zoonotic reservoirs from which infections may be transferred to humans (Olson et al., 1997; Olson et al., 2004; Keeley and Faulkner, 2008).

### 2.7.1 CRYPTOSPORIDIUM ZOONOTIC TRANSMISSION

The existence of host-adapted *Cryptosporidium* species suggests that cross transmission of *Cryptosporidium* between different animal groups is limited. However, given that animals may share the same habitat combined with the fact that parasite species are capable of infecting multiple hosts, cross transmission can occur. Advances using molecular epidemiological testing have resulted in the detection of the following zoonotic *Cryptosporidium* species in sheep faeces; *C. parvum*, *C. hominis*, *C. andersoni*, *C. suis*, and *C. ubiquitum* (Fayer, 2008; Xiao and Fayer, 2008). Yang *et al.* (2009) identified all of these potentially zoonotic species in pre- and post-weaned lambs in WA. The species, *C. parvum* (Sulaiman *et al.*, 1998; Harper *et al.*, 2002; Ng *et al.*, 2008; Ng *et al.*, 2010a; Ng *et al.*, 2010b), *C. hominis* (Park *et al.*, 2006; Ng *et al.*, 2010a; Ng *et al.*, 2010b), *C. andersoni* (Morse *et al.*, 2007) and *C. ubiquitum* (Ong *et al.*, 2002; Wong and Ong, 2006; Chalmers *et al.*, 2009) have all been identified as being able to infect humans and they also possess zoonotic capability.

It has been reported that *C. parvum* is a significant *Cryptosporidium* species present in young livestock (Xiao *et al.*, 2002; Fayer *et al.*, 2007; Mueller-Doblies *et al.*, 2008; Santin *et al.*, 2008; Yang *et al.*, 2009; Fayer *et al.*, 2010a). In beef and dairy cattle, *C. parvum* is
considered prevalent in young calves less than 2 months old, with older calves and mature cattle more commonly infected by *C. bovis* and *C. andersoni* (Fayer *et al.*, 2007; Santin *et al.*, 2008; Fayer *et al.*, 2010a; Ng *et al.*, 2011a). Recent studies on sheep in Australia and the USA have reported *C. parvum* oocysts in sheep faeces (Ryan *et al.*, 2005; Santin *et al.*, 2007; Yang *et al.*, 2009), with just under half of the lamb samples screened for *Cryptosporidium* in a recent study confirmed as *C. parvum*-positive (Yang *et al.*, 2009). It has been reported that physical contact with domestic livestock, particularly cattle, increases the risk of contracting cryptosporidiosis (Robertson *et al.*, 2002; Hunter *et al.*, 2004; Olson *et al.*, 2004; Xiao, 2010; Yoder and Beach, 2010). Recent beef cattle studies in Canada (Dixon *et al.*, 2011) and India (Khan *et al.*, 2010) have confirmed zoonotic transmission and potentially also zoanthroponotic transmission. Contact with *Cryptosporidium*-infected calves has been implicated as the cause of small cryptosporidiosis outbreaks in veterinary students, animal researchers and members of the general public who have attended agricultural shows and fairs (Ashbolt *et al.*, 2003; Preiser *et al.*, 2003; Smith *et al.*, 2004; Kiang *et al.*, 2006). In addition, studies in both the UK and USA have found livestock faecal pollution of water sources to be a leading cause of human cryptosporidiosis (Zhou *et al.*, 2003; Goh *et al.*, 2004; Feng *et al.*, 2007; Yang *et al.*, 2008; Smith and Nichols, 2010).

### 2.7.2 GIARDIA ZOONOTIC TRANSMISSION

The existence of animal to human zoonotic transmission has been documented with two food borne outbreaks of giardiasis caused by rat and sheep faeces (Smith *et al.*, 2006; Feng and Xiao, 2011). The two *Giardia* genotype assemblages A and B infect both humans and animals and pose the greatest zoonotic risk to human public health (Geurden *et al.*, 2010b; Feng and Xiao, 2011). Yang *et al.*, (2010) reported potential zoonotic transmission...
in human cases in WA. Research in Victoria, Australia by Nolan et al. (Nolan et al., 2010) found a high prevalence of assemblage A in lambs, with one dominant sequence which had been isolated from humans. The human pathogenic assemblages A and B are often at low prevalence levels, usually competing with the more common assemblage E (Thompson and Monis, 2004; Castro-Hermida et al., 2007; Santin et al., 2007; Robertson et al., 2010). Aloisio et al. (2006) however reported a high prevalence of assemblage B in Italian sheep, although this was concluded from a PCR analysis of only 2 samples.

Case control studies in New Zealand have found that human contact with pets was not associated with an increased risk of giardiasis, although contact with farm animals was associated with an increased risk (Hoque et al., 2002; Hoque et al., 2003). Human giardiasis infection rates were reported to be 23% higher in rural than urban areas (Snel et al., 2009). This was further supported by an increased giardiasis prevalence, when humans were in contact with farm animals (particularly pigs, sheep and cattle) in England and potential zoonotic transmission existed in a Canadian dairy enterprise. However, studies in the USA don’t support this association (Xiao and Fayer, 2008; Dixon et al., 2011). As with Cryptosporidium, studies involving Giardia transmission through water contamination have been conducted worldwide. These studies have demonstrated that livestock faecal pollution of water sources is a major cause of human giardiasis (Rose et al., 1991; Medema et al., 1998; Olson et al., 1999; Wilkes et al., 2009; Ng et al., 2011b)

2.8 EIMERIA INFECTIONS

Coccidial infections in sheep have been observed in almost all sheep rearing countries of the world (Pellérdy, 1974; Taylor et al., 2003; Wang et al., 2010a; Taylor et al., 2011), with the assumption that most domestic ruminants contract coccidian infections
during some stage of their lives (Foreyt, 1990; Taylor and Catchpole, 1994; Platzer et al., 2005). Coccidiosis in sheep is caused by the protozoan parasite *Eimeria* and in the majority of animals, the parasite co-exists with the host causing minimal damage (O'Callaghan et al., 1987; Platzer et al., 2005). Sheep of all ages are susceptible, although disease outbreaks are typically observed in young lambs 1–3 months old (2–3 weeks after weaning) following an incubation period of ~14–20 days (Platzer et al., 2005; Taylor et al., 2011) or when sheep are housed in barns or feedlots (O'Callaghan et al., 1987; Foreyt, 1990; Taylor and Catchpole, 1994; Coop and Wright, 2000). In Australia, 15 different species of *Eimeria* have been isolated in healthy sheep; the most significant of these include *E. crandallis/weybridgeensis*, *E. ovina*, *E. ovinoidalis*, *E. granulosa*, *E. parva/pallida*, *E. intricata*, *E. ahsata*, *E. faurei* and *E. punctata* (O'Callaghan et al., 1987; Foreyt, 1990; Platzer et al., 2005).

Of these *Eimeria* spp., *E. ovinoidalis* and *E. crandallis* are considered pathogenic and cause the most severe disease, with coccidial infections commonly appearing asymptomatic with multiple *Eimeria* spp. present (Gregory and Catchpole, 1987, 1990; Taylor et al., 2003). The different species vary in their preferential location along the gastrointestinal tract in the large intestine, as a result of which the large intestine is unable to counteract disruption and damage to the proximal gut regions. This is manifest by an increased water and electrolyte digesta content, increasing the risk of diarrhoea and impairing both food and water absorption (Gregory and Catchpole, 1987; Glastonbury, 1990; Gregory and Catchpole, 1990; Taylor and Catchpole, 1994; Taylor et al., 2011). Increased stocking rates of young lambs on pasture has contributed to coccidiosis becoming a significant problem, particularly in the UK in twin born lambs 3–8 weeks old (Taylor and Catchpole, 1994) and in France 2–3 weeks old (Yvoré and Esnault, 1987).
After bouts of such early infection, animals develop resistance to coccidia as a result of an acquired immunity. They are not capable of excluding all parasites, but are capable of preventing clinical disease (Taylor and Catchpole, 1994; Taylor et al., 2011).

2.8.1 LIFE CYCLE AND TRANSMISSION

The life cycle of this *Eimeria* spp. has both asexual and sexual development cycles occurring within the one host. Following ingestion of oocysts, the parasites multiply several times, destroying host tissue, before producing oocysts that are shed in faeces (Taylor et al., 2011). Two major stages of asexual development occur within this life cycle; one which produces merozoites and sporozoites (Figure 2.11) (Entzeroth et al., 1998; Ng et al., 2002). An additional extra-intestinal, asexual phase of multiplication called endodyogeny can occur, although it is not essential for completion of the life cycle. The intracellular life cycle stages of *Eimeria* parasites include; merozoites, schizonts (meronts) and gametocytes, which are all located within the host cell in a membrane bound parasitophorous vacuole. However, sporogony typically occurs outside the host (Figure 2.11) (Entzeroth et al., 1998). Microscopy and PCR are the known techniques for identification of *Eimeria* spp. in faecal samples (Kayla, 1998; Haug et al., 2007).
Figure 2.1: Left – Diagrammatic representation of the life cycle of *Eimeria* in an infected mammal. Right – Illustration of a sporulated oocyst (a), sporocyst (b) and sporozoite (c) of *Eimeria*. Adapted from Entzeroth *et al.*, (1998), Sam-Yellowe (1996) and Lindsay and Tood (1993).

### 2.8.2 CLINICAL SIGNS, PATHOLOGY AND PRODUCTION LOSS

*Eimeria* are important parasites which commonly infect domestic livestock and have special significance from a clinical perspective when animals are held in feedlots, barns or animal houses, where stressful, confined conditions can contribute to poor growth rates and diarrhoea (Gregory and Catchpole, 1987, 1990; Reid, 1990; Taylor and Catchpole, 1994; Taylor *et al.*, 2011). Following infection, immunity to the parasite develops and older sheep are generally resistant to any pathogenic effects (Foreyt, 1990; Radostits *et al.*, 1994; Platzer *et al.*, 2005; Taylor *et al.*, 2011). Acute infections cause disturbance of the gastrointestinal tract, which leads to the discharge of water, electrolytes and protein, with diarrhoea one clinical sign of acute coccidiosis. By comparison, sheep suffering from a chronic coccidiosis infection, will suffer malabsorption challenges and diarrhoea due to
proliferative lesions (“polyps”). Pathophysiological disruption to the gastrointestinal tract causes villous atrophy, defects in absorptive cells of the gut epithelium and impaired nutrient transport from the epithelial lining to an adjoining blood repository (Gregory and Catchpole, 1987; Foreyt, 1990; Gregory and Catchpole, 1990; Reid, 1990; Taylor and Catchpole, 1994; Taylor et al., 2011). Further clinical signs associated with coccidiosis include blood or intestinal casts, ill-thrift, weight loss, abdominal pain, anaemia and deaths (Gregory and Catchpole, 1987, 1990; Taylor, 1995; Navarre and Pugh, 2002; West et al., 2002; Taylor et al., 2003). Multiple factors affect the prevalence of clinical Eimeria (coccidiosis) infections; these include which species of Eimeria are present, the level of passive and acquired immunity in sheep, the presence of other enteric infections, poor hygiene and the stress associated with overcrowding, transport, inclement weather and poor nutrition (Gregory and Catchpole, 1987; Foreyt, 1990; Gregory and Catchpole, 1990; Platzer et al., 2005).

2.8.3 TREATMENT

Metaphylactic treatment has been documented as a successful management option, used to prevent coccidiosis in calves, although both the 2–3 weeks during PPRI and the 3–10 days of Eimeria spp. oocyst sporulation in the environment, contribute to an increased environmental infection exposure risk (Taylor et al., 2003; Daugschies et al., 2007; Taylor et al., 2011). Diclazuril (Vecoxan® Janssen Animal Health) has been shown to be effective in treating lambs and calves infected with Eimeria species. This chemotherapeutic agent has a direct effect on several stages of E. crandallis life cycle in lambs, specifically the large first stage meront (Taylor et al., 2003; Daugschies et al., 2007; Taylor et al., 2011) and is effective within four days following administration (0.25–1.0mg/kg live weight), although it is less effective on second generation meronts and gametocytes. The diclazuril
treatment was most effective when administered in the early stages of infection in young lambs raised on pastures, before significant damage to the gastrointestinal tract had occurred (Taylor et al., 2003; Daugschies et al., 2007; Taylor et al., 2011). The treatment also fostered the development of protective immunity in young lambs, thereby protecting them against any subsequent heavy coccidial challenge (Taylor et al., 2011).

2.9 BACTERIAL INFECTIONS

Along with internal parasites, there are several species of bacteria considered to be contributing factors towards outbreaks of gastroenteritis and diarrhoea in sheep in WA. Of these bacteria, the most important species are reviewed below (Table 2.4). For bacterial infections that contribute to diarrhoea in sheep, there are some important points to consider relating to their virulence. Firstly, each species of bacteria responsible for diarrhoea often occurs naturally in sheep. For clinical signs of the bacterial infection to be observable, sheep are required to be exposed to different forms of stress. Some of the factors which contribute to stressful conditions leading to gastroenteritis and that provide clinical signs of bacterial infections include poor weather conditions, overcrowding, confinement, fatigue, other pathogenic infections, nutritional challenges and changes in feed or husbandry management practices. As with internal parasites associated with diarrhoea in sheep, bacterial infections are most often observed in young lambs (except with Mycobacterium infections), while adult sheep at least 2 years old are unlikely to have production consequences from bacterial infections. Finally, with respect to bacterial gastroenteritis, sheep often display clinical signs that are associated with many other pathogens, including bacteria. Diagnosis of these bacterial infections are traditionally conducted via culturing, histopathological examinations, immunoblotting, blood antibody tests, ELISA and PCR (Bolton et al., 1984; Corry et al., 1995; Denis et al., 1999; Oliveira et al., 2002; Whyte et al.,
2002; Diergaardt et al., 2004; Wilkes et al., 2009; Pusterla et al., 2010; Tatavarthy and Cannons, 2010).
Table 2.4: The major bacteria associated with diarrhoea and production losses in extensively grazed lambs.

<table>
<thead>
<tr>
<th>Bacteria family</th>
<th>Species</th>
<th>Transmission</th>
<th>Pathology</th>
<th>Clinical signs</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmonella</td>
<td>S. enterica, S. typhimurium, S. bovis-morbificans, S. arizona, S. dublin, S. havana, S. enteritidis, S. abortus</td>
<td>Carrier sheep not displaying clinical signs of infection.</td>
<td>Necrosis and inflammation, particularly in the liver.</td>
<td>Diarrhoea (often with blood specks and a putrid odour) septicaemia weight loss, depression, dehydration, fever, fatigue, abortion and sudden death.</td>
<td>(Plagemann, 1989; Wray et al., 1991; Nesbakken, 2009; Duffy et al., 2010; Tatavarthy and Cannons, 2010)</td>
</tr>
<tr>
<td>Campylobacter</td>
<td>C. jejuni, C. coli</td>
<td>Carrier weaner sheep not displaying clinical signs of infection.</td>
<td>Disruption of the microvilli, exudative superficial erosive colitis and toxin release.</td>
<td>Diarrhoea (often a dark green liquid), septicaemia, weight loss, depression, dehydration, fever, fatigue and sudden death.</td>
<td>(Friedman et al., 2000; Diergaardt et al., 2004; Wilkes et al., 2009)</td>
</tr>
<tr>
<td>Mycobacterium</td>
<td>C and S strains of Mycobacterium avium subsp. Paratuberculosis</td>
<td>Carrier sheep not displaying clinical signs of infection.</td>
<td>Chronic antigenic stimulation from M. paratuberculosis residing in intestinal tissues. Cell mediated response replaced by antibody mediated responses.</td>
<td>Progressive weight loss, emaciation, diarrhoea (soft faeces rather than liquid).</td>
<td>(Seaman et al., 1981; Seaman and Thompson, 1984; Sharp, 2000; Whittington et al., 2000; Stewart et al., 2004; Whittington, 2004; Stewart et al., 2006; Dhand et al., 2007; Zhong et al., 2011).</td>
</tr>
<tr>
<td>Listeria</td>
<td>L. monocytogenes</td>
<td>Contaminated feed, particularly silage and carrier sheep not displaying clinical signs of infection.</td>
<td>Necrosis of intestinal mucosa of both the small and large intestine</td>
<td>Diarrhoea (brown liquid), encephalitis, abortion, septicaemia and sudden death.</td>
<td>(Olson et al., 1953; Low and Donachie, 1997).</td>
</tr>
<tr>
<td>Yersinia</td>
<td>Y. pseudotuberculosis, Y. enterocolitica, Y. intermedia, Y. frederiksenii.</td>
<td>Carrier weaner sheep not displaying clinical signs of infection.</td>
<td>Necrosis of mesenteric lymphatics and abscesses of liver and lung</td>
<td>Diarrhoea (often a green, black colour often mucoid or blood specked), weight loss, depression, dehydration, fever, fatigue, abortions.</td>
<td>(McSporran et al., 1984; Buddle et al., 1988; Slee and Button, 1990; Bin-Kun et al., 1994; Juste et al., 2009; Okwori et al., 2009).</td>
</tr>
<tr>
<td>Clostridium</td>
<td>C. perfringens type D</td>
<td>Often due to an increased availability of rapidly fermentable carbohydrates following sheep introduced to grain, cereal stubbles or lush pastures.</td>
<td>Haemorrhagic enteritis of the small intestines due to enterotoxins and/or cytotoxins</td>
<td>Diarrhoea, weight loss, depression, anorexia, neurological signs, paralysis, bloat and sudden death.</td>
<td>(Niilo, 1980; Radostits et al., 1994; Lewis, 2000; Uzal and Songer, 2008).</td>
</tr>
</tbody>
</table>
2.9.1 SALMONELLA

Salmonella usually occur in small numbers in the gastrointestinal flora of healthy sheep. Salmonellosis has been reported on sheep farms and abattoirs worldwide (Smith, 1996; Zweifel et al., 2004; Woldemariam et al., 2005; Belloy et al., 2009; Wilkes et al., 2009; Duffy et al., 2010) and is believed responsible for economic losses in some sheep enterprises (Plagemann, 1989; Wray et al., 1991). Salmonella is typically an acute, sporadic disease and is typically associated with very high mortalities being observed during infection outbreaks (Table 2.4) (Glastonbury, 1990; Linde et al., 1992; Radostits et al., 1994; Smith, 1996; Woldemariam et al., 2005; Andrés et al., 2007).

2.9.2 CAMPYLOBACTER

This bacterial infection is due predominantly to the presence of Campylobacter jejuni, which can cause enterocolitis in sheep within Australia. Campylobacteriosis is commonly referred to as ‘weaner colitis’ and is typically observed in weaner lambs (1–3 months of age). (Skirrow, 1994; Friedman et al., 2000; Zweifel et al., 2004; Olson et al., 2008; Sahin et al., 2008; Sheppard et al., 2009; Garcia et al., 2010). The significant pathophysiological consequences of Campylobacter infections include decreased absorption in the colon resulting from a disruption to the microvilli and exudative superficial erosive colitis (Table 2.4). The mechanism by which Campylobacter cause the above, is by attachment to the wall of the gastrointestinal tract and subsequent release of toxins (enterotoxins and/or cytotoxins) (Glastonbury, 1990; Sargison, 2004; Olson et al., 2008).

2.9.3 OVINE JOHNE’S DISEASE

Paratuberculosis or Ovine Johne’s disease (OJD), was first recorded in sheep in Australia in 1980 (Seaman et al., 1981; Whittington et al., 2000; Stewart et al., 2004, 2006;
Dhand et al., 2007). Mortalities and subsequent economic losses vary considerably among infected sheep farms, with a study by Bush et al. (2006) indicating an average reduction in income of $13,715 per farm per year for 12 infected Merino sheep flocks. Western Australia was declared free of OJD until 2003, when it was detected in several properties throughout WA and two of these WA properties were included in a study of OJD risk factors (Dhand et al., 2007). Ovine Johne’s disease is commonly referred to as ‘the wasting disease’, due to a progressive weight loss. The clinical signs of the disease (Table 2.4) are typically observed in adult sheep older than two years, rather than in young weaner lambs, as the disease has a prolonged, variable incubation period and is usually manifest as a chronic infection (Seaman and Thompson, 1984; Dhand et al., 2007).

2.9.4 **LISTERIA**

Listeriosis is an important bacterial zoonosis caused by *Listeria monocytogenes* and usually occurs following the ingestion of contaminated food and is particularly common in ruminants fed silage (Table 2.4) (Olson et al., 1953; Low and Donachie, 1997). Listeriosis in sheep is a concern for producers and a public health risk, particularly in feedlot nutrition mixes (Blood and Radostits, 1994; Nash et al., 1995).

2.9.5 **YERSINIA**

Ovine yersiniosis has been reported to be associated with diarrhoea outbreaks and abortions in the United Kingdom (Table 2.4) (Corbel et al., 1990; Corbel et al., 1992). It emerged in the 1990’s in south-eastern Australia and New Zealand, although an outbreak has not been reported in WA.
2.9.6 CLOSTRIDIUM

*Clostridium perfringens* type D produces a disease in sheep, often referred to as ‘pulpy kidney disease’ or enterotoxaemia. *Clostridium perfringens* produces alpha and epsilon toxins, which can cause minor clinical signs of diarrhoea (Layana *et al.*, 2006; Uzal and Songer, 2008). The clinical signs are often not detected in young lambs, as the illness is acute, lasting no longer than 12 hr, while adult sheep affected by enterotoxaemia may survive for up to 24 hr (Niilo, 1980; Radostits *et al.*, 1994; Lewis, 2000; Uzal and Songer, 2008). Diagnosis of this disease in sheep is generally associated with previous animal history (medical treatments and vaccinations), typically occurring in unvaccinated sheep and those which are fed a high risk diet of rapidly fermentable carbohydrates (Table 2.4).

2.9.7 CHLAMYDOPHILA

*Chlamydophila pecorum* is a bacteria species from the Chlamydiaceae family, which has been isolated from ruminants, marsupials and pigs, although there are no reports of this bacteria isolated from sheep in Australia. The major clinical signs associated with infection include abortion, infertility and urinary tract infection and is a public health risk to pregnant women (McCauley *et al.*, 2010; Pantchev *et al.*, 2010).

2.10 VIRAL INFECTIONS

In WA there are no known viruses that cause diarrhoea in lambs post-weaning. In young lambs up to 10 weeks of age, viruses from the rotavirus and pestivirus genus may cause diarrhoea and ill-thrift, although these viruses do not cause diarrhoea in older sheep (Vilcek *et al.*, 1997). There are two virus types of pestivirus, both from the Flaviviridae family that may cause diarrhoea in sheep. These include the border disease virus and bovine viral diarrhoea virus I and II (Radostits *et al.*, 1994; Vilcek *et al.*, 1997). There are
many other viruses exotic to Australia that are associated with diarrhoea. These viruses include Rift Valley fever, Rinderpest, Peste des petitis and Nairobi sheep disease (Napthine, 1988; Farquarson, 1992; Blood and Radostits, 1994).

### 2.11 NON-PATHOGENIC FACTORS INFLUENCING LAMB PRODUCTION PERFORMANCE AND FAECAL ATTRIBUTES

In order to investigate the impact that internal parasites have on lamb production performance (i.e. growth rate, carcase weight and condition score) and diarrhoea in sheep, it is important to emphasise that other non-pathogenic factors also influence both production and diarrhoea. These factors include breed of sheep, sex, genetics, environment, climate, stock management and husbandry, lambing time and nutrition (Abbott et al., 1986; Suiter and Morris, 1986; Ritar et al., 1990; Bown et al., 1991a; Bowman et al., 1995; Coop and Sykes, 2002; Kahn et al., 2003; Cloete et al., 2004; Liu et al., 2005; Louvandini et al., 2006; Houdijk, 2008).

Sheep faecal matter consists of undigested cell walls, carbohydrates, microbial debris and water (Van Soest, 1994; Waghorn et al., 1999) from the different forms of feed they consume. The composition of faeces is influenced predominantly by two factors, diet digestibility and the amount of feed intake (Wesselink et al., 1995), with digestibility decreasing as intake is increased (Fahey and Hussein, 1999). The water content of the digesta is predominantly influenced by sheep diet (Waghorn et al., 1999). A high digesta water content and rapid flow rate through the small and large intestines, hinders the effective absorption of water and minerals, resulting in either poor or no faecal pellet formation in the spiral colon (Reid and Cottle, 1999; Waghorn et al., 1999).
2.12 LITERATURE REVIEW CONCLUSION

Internal parasitism reduces the profitability of sheep enterprises worldwide. Reduced production performances and increased treatment costs are typically associated with the occurrence of strongylid worms in Australian sheep enterprises (Love and Coles, 2002; Besier and Love, 2003; Sackett *et al.*, 2006).

Little information exists regarding the effects that the protozoan parasites; *Cryptosporidium*, *Giardia* and *Eimeria*, have on meat lamb production performances. It is likely that these protozoa are collectively one of many multiple factors that potentially can contribute to production losses, reduced carcase profitability and pose an increased risk for incidences of diarrhoea in young, growing lambs. This knowledge gap has justified further investigation involving on-farm and abattoir studies in WA. These studies will examine the impacts that both strongylid nematodes and protozoa have on lamb live weight, growth rate, BCS, breech fleece faecal soiling, carcase attributes and faecal attributes.

These protozoa are an evolving issue for ruminant enterprises, particularly due to the potential zoonotic risk they pose and their potential to contaminate human drinking water sources. A thorough investigation is necessary to examine if these protozoa isolated from sheep, are a potential zoonotic risk to public health and to assess whether in isolation or together with strongylid nematodes, they are associated with production performances and the risk of diarrhoea in lambs.

It is possible that identification of *Cryptosporidium* and/or *Giardia* in lambs on one or more occasions will result in a significant reduction in carcase weight, dressing percentage, live weight and growth rate. Furthermore, as little investigation has been conducted to examine what pathogenic associations between different species/genotypes of
Cryptosporidium and Giardia and their impact upon live weight, growth rate, BCS, carcase attributes or faecal attributes, analyses of these characteristics will increase knowledge of sheep growth parameters.

One explanation, albeit simplistic, is that associations may exist between individual internal parasites and both production and diarrhoea attributes in meat lambs. However it is more probable that complex relationships exist between all these attributes and when combined with sheep parasite status (whether lambs are positive for mixed internal parasite infections), they are likely to be key determining factors. In order to estimate the overall consequences protozoa may have on meat lamb production, a variety of different farm locations were selected. At these farms, individual lambs were identified shortly following their birth, and monitored throughout their life on pasture until their slaughter at commercial abattoirs.

2.10 PROJECT AIMS

1. Provide survey information on the prevalence, severity and proportion of lamb flocks which display signs of active diarrhoea or where recent evidence of diarrhoea (breach fleece faecal soiling) exists in meat lamb enterprises, whilst also examining possible risk factors associated with diarrhoea. Further information is provided regarding the frequency with which enterprises conduct WECs, whether anthelmintics are administered to pregnant ewes, weaner meat lambs or both and finally an indication regarding the level of awareness amongst farm managers of internal parasites, other than strongylid worms that cause sheep disease in their district.
2. To determine if recently developed species-specific strongylid PCR assays can successfully amplify genomic DNA extracted directly from faeces, by utilising a commercial DNA isolation kit. These results from strongylid diagnostic PCR assays will be compared to traditional McMaster WEC microscopy results to test the level of agreement between these two diagnostic techniques.

3. Investigate if a modified molecular identification method can be implemented to detect and potentially quantify DNA extracted directly from material (pasture, debris and larvae) recovered using a modified strongylid larvae recovery procedure from pasture.

4. To compare PCR, qPCR and McMaster WEC results to determine if there is an association between molecular and WEC parasite results.

5. To ascertain whether protozoan prevalence and WECs from faecal sampling lambs in lairage is representative of the shedding for both strongylid and protozoan parasites on-farm.

6. To establish if lambs that are PCR-positive for protozoan parasites have an increased risk of diarrhoea and suffer greater production losses, when compared to those lambs that are protozoa-negative.

7. To examine if different species/genotypes of Cryptosporidium and Giardia are associated with an increased risk of diarrhoea and greater production losses.

8. To present estimations of the production consequences that strongyloid and protozoan infections may have on meat lamb productivity and profitability.

9. To contrast the production performances of two lamb flocks grazing separate paddocks that have different natural strongylid L₃ levels on pastures.
10. To hypothesise potential farm management and sheep husbandry practices, which help minimise both production losses and the risk of diarrhoea outbreaks associated with protozoan infections in meat lambs.

2.10 HYPOTHESES

1. Diarrhoea is widespread in lamb flocks throughout all agricultural zones.
2. The majority of surveyed respondents are unaware that Cryptosporidium and Giardia are capable of causing disease in sheep.
3. For patent strongylid infection detection, the levels of agreement between modified McMaster WEC and molecular PCR results are low.
4. Lambs classified as PCR-positive for Cryptosporidium and/or Giardia on at least one sampling occasion have reduced carcase attributes (hot carcase weight [HCW], dressing percentage, GR fat depth and fat score) when compared to those lambs never positive for either protozoan species.
5. Lambs classified as PCR-positive for Cryptosporidium and/or Giardia have reduced production attributes (live weight, growth rate and BCS) when compared to those lambs protozoan negative.
6. Lambs with a high number of internal parasites detected (both protozoan and strongylid species) have BCSs and FCSs.
7. Lambs classified as PCR-positive for protozoa are of greater risk of having more loose, non-pelleted faeces, along with higher FCSs, lower FDM%ks and higher breech fleece faecal soiling scores, when compared to negative lambs.
8. Lairage WECs and protozoa prevalences are not representative of the shedding of these parasites on-farm.
9. The more samplings lambs test PCR-positive for *Cryptosporidium* and/or *Giardia*, the greater reduction in HCW and dressing percentage, when compared to those lambs which are PCR-positive for either protozoon on only one occasion or never at all.

10. Lambs exposed to a higher natural strongylid L₃ challenge have lighter carcase yields, lower dressing percentages, reduced productivity performances (live weight, growth rate and BCS) and more loose, non-peletted faeces, when compared to lambs exposed to a lower strongylid L₃ challenge.
CHAPTER 3: MATERIALS AND METHODS

3.1 HUMAN AND ANIMAL ETHICS APPROVAL

The Murdoch University Human Research Committee approved the mail survey described in Chapter Four (HREC permit number 2009/222). The research described in Chapters Five through to Ten was approved by the Animal Ethics Committee (AEC) under the following AEC permits R2236/09 and R2369/10. All sheep were handled according to the Code of Practice for Sheep in Western Australia (Department of Local Government and Regional Development Western Australia, 2003).

3.2 CHOICE OF SAMPLING LOCATIONS

The sampling locations were selected across southern Western Australia to identify the prevalence of both protozoa and strongylid nematode species in meat lamb flocks. Choices of these locations were largely dependent on the enterprise manager willing to participate in this research project, with nominated enterprises having meat lamb production as part of their overall sheep enterprise. Basal information for the sheep enterprises (i.e. farm size, winter stocking rate, livestock water source, etc) sampled in this study is shown in Table 3.1 and the geographical location in Figure 3.1.

The first sheep property sampled was located ~210km south east of Perth within ~20km of the town Pingelly, with an annual rainfall of 400–450mm. The second property was ~225km south east of Perth, situated ~10km from Arthur River, with an annual rainfall of 450–500mm. Data from the Pingelly and Arthur River enterprises are presented in Chapters Five, Six, Seven and Nine.
Two different sheep meat enterprises in southern Western Australia were selected for sampling and were part of a project run in conjunction with the Department of Agriculture and Food, Western Australia. The first property was located within ~50km of the town Boyup Brook, with an annual rainfall of 550mm. The second property was located within ~20km of the town of Kojonup and had an annual rainfall of 500–550mm. Boyup Brook and Kojonup data are presented in Chapters Five and Eight.

For the final study, a separate meat lamb enterprise located ~360km south, south east of Perth, within ~10km Frankland, was selected. This enterprise had an annual rainfall of 550–600mm, although in recent years annual rainfall has been less than 500mm. Frankland data are presented in Chapter Ten.

The final carcase measurements and lairage faecal samplings were conducted in three commercial abattoirs; the first was 6km north of Narrogin (Hillside Tender Meats abattoir), the second was 50km north of Albany at Narrikup (Fletchers International abattoir) and the third was 100km east of Northam (Tammin abattoir, Tammin) (Figure 3.1).
Table 3.1: Sampling locations and farm information for chosen meat lamb enterprises.

<table>
<thead>
<tr>
<th>Farm location</th>
<th>Mean annual rainfall</th>
<th>Farm size</th>
<th>Number of sheep</th>
<th>Lamb breed</th>
<th>Commencement of Lambing</th>
<th>Goats and/or cattle on property</th>
<th>Livestock water source</th>
<th>Winter stocking rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pingelly</td>
<td>450mm</td>
<td>1500Ha</td>
<td>1350</td>
<td>Merino x Suffolk</td>
<td>Mid July</td>
<td>No</td>
<td>Creek</td>
<td>12 DSE/Ha</td>
</tr>
<tr>
<td>Arthur River</td>
<td>500mm</td>
<td>1250Ha</td>
<td>1750</td>
<td>Merino x Suffolk</td>
<td>Early August</td>
<td>No</td>
<td>Dam</td>
<td>10 DSE/Ha</td>
</tr>
<tr>
<td>Boyup Brook</td>
<td>450mm</td>
<td>1500Ha</td>
<td>1350</td>
<td>Merino x White</td>
<td>Mid July</td>
<td>Cattle, no goats</td>
<td>Dam</td>
<td>5.6 DSE/Ha</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Suffolk</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kojonup</td>
<td>550mm</td>
<td>1250Ha</td>
<td>1750</td>
<td>Merino x Poll</td>
<td>Early August</td>
<td>No</td>
<td>Dam</td>
<td>9 DSE/Ha</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Dorset</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frankland</td>
<td>550mm</td>
<td>560Ha</td>
<td>3300</td>
<td>Merino x Suffolk</td>
<td>Mid July</td>
<td>No</td>
<td>Dam</td>
<td>21 DSE/Ha</td>
</tr>
</tbody>
</table>

Note: DSE = Dry Sheep equivalent, is a standard unit frequently used to compare animal carrying capacity and potential productivity of a given farm or area of grazing land. It also aids in helping assess feed requirements of different classes for livestock (McLaren, 1997).
3.3 ANIMALS

On both the Pingelly and Arthur River properties, Merino ewes between 2–4 years of age were selected and joined (mated) with Suffolk rams. The lambs from these Merino ewes were Merino x Suffolk lambs, also commonly referred to as ‘cross lambs’. Merino ewes between 2–4 years old on the Boyup Brook property were joined with White Suffolk rams to produce Merino x White Suffolk lambs. For the Kojonup property, Merino ewes ~4 years old were joined with Poll Dorset rams to produce Merino x Poll Dorset lambs. On the
Frankland farm, Merino ewes ~6 years old were selected and joined with Suffolk rams, producing Merino x Suffolk lambs (Table 3.1).

All lambs in these research studies were raised specifically for slaughter, with each lamb randomly selected and identified with a uniquely numbered ear tag and a radio-frequency ear tag. Individual lamb flocks were raised on a specific paddock, with annual rye-grasses (*Lolium* spp.) and subterranean clover (*Trifolium subterraneum*) the major pasture plant species. Water was supplied to the lambs *ad libitum*.

### 3.4 FAECAL SAMPLES

On the Pingelly, Arthur River and Frankland properties, preliminary faecal samples were collected from pregnant ewes. The first occasion was within four months of the ewes commencing lambing and the second ~2 weeks before the ewes were due to lamb, in order to determine the prevalence of protozoa and strongylid species within each flock.

When the lambs were ~2–6 weeks old, female lambs from each of these three properties were randomly selected and identified with a uniquely numbered ear tag and a radio-frequency ear tag. Faeces were collected directly from the rectum of only these identified female lambs, with faecal samples collected from the same lambs on five different occasions; between the first (2–6 weeks of age) and final (7–8 months old) samplings.

On the Boyup Brook and Kojonup properties, lambs from each flock were randomly selected and identified with ear tags when ~2–6 weeks old. Faecal samples were collected from these lambs on two occasions; the first occasion when they were 2–3 months old and the final time when they were 4–5 months old.
Each flock was mustered from their paddock into nearby yards for faecal sampling, except when sampling took place in lairage, following the transport of lambs by road to the abattoir from their respective farms. For all faecal samples collected, disposable latex gloves were used in collecting and handling each sample to avoid cross contamination. All faecal samples were collected from the rectum of lambs. All faecal samples were placed in individually labelled, airtight 70 mL containers with the air expressed and stored at 2–4°C before processing.

### 3.5 Faecal Consistency Score

Faeces in sheep vary in consistency, ranging from those which are firm faecal pellets and are a characteristic of healthy sheep, faecal pellets which are losing their form and compressed into stools, faeces that are formed in stools, semi-watery/pasty faeces and liquid diarrhoea (Greeff and Karlsson, 1998; Waghorn et al., 1999; Le Jambre et al., 2007). Faecal consistency score was measured using a scale of 1–5 (Table 3.2 and Figure 3.2).

Table 3.2: Criteria used in assessment of faecal consistency score (FCS).

<table>
<thead>
<tr>
<th>Faecal consistency score</th>
<th>Faecal consistency description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hard, dry pellet, cracks when placed under firm pressure between finger and thumb</td>
</tr>
<tr>
<td>2</td>
<td>Soft, moist pellet, less tendency to crack when placed under firm pressure between finger and thumb</td>
</tr>
<tr>
<td>3</td>
<td>Soft faecal mass, loss of pellet structure</td>
</tr>
<tr>
<td>4</td>
<td>Soft paste, pasty diarrhoea</td>
</tr>
<tr>
<td>5</td>
<td>Liquid/fluid</td>
</tr>
</tbody>
</table>
Figure 3.2: Graphical representation of differing faecal consistency scores (FCSs) (Dalton, 2006).
3.6 FAECAL DRY MATTER

Faecal dry matter analyses were performed following the collection of fresh faeces that were stored in air tight containers for ~3 days, as recommended by the Association of Official Analytical Chemists (AOAC) (AOAC, 1997). Briefly, 80mL test tubes were labelled individually and stored in a dry oven at 105 °C overnight. Following the initial labelling and drying of test tubes, the net weight of each test tube was weighed, with care taken to use disposable latex gloves (to prevent adding moisture to dried faecal samples). From each faecal sample, 0.5–1g of faeces was added to each tube, which was then weighed again to record the ‘wet weight’. All samples were left in the dry oven at 105 °C for 48 hr. Each sample (test tube and dried faeces) was then weighed again while using disposable latex gloves to determine the ‘dry weight’. This analysis was duplicated, with a second 0.5–1g of sample of faeces taken from each lamb faecal sample following the same protocol above. Faecal dry matter percentage was calculated (Equation 3.1) and an average of the two FDM% measurements was calculated to provide the final FDM%.

**Equation 3.1:** Faecal dry matter percentage (FDM%) calculation from fresh faecal samples.

\[
Dry \text{ Matter Percentage} \ (\%) = \frac{\text{Dry weight (g)} - \text{beaker weight (g)}}{\text{Wet weight (g)} - \text{beaker weight (g)}} \times 100
\]

3.7 BREECH FLEECE FAECAL SOILING SCORE

The extent to which faecal soiling adhered to the breech of each lamb was assessed subjectively using a breech fleece faecal soiling score scale ranging from 1–5; with a score one having no visible breech fleece faecal soiling, to a score five having extensive fleece...
faecal soiling in the breech area, but also extending right down the hind legs to the pasterns (Australian Wool Innovation et al., 2007).

Figure 3.3: Graphical representation of the breech fleece faecal soiling scale (Australian Wool Innovation et al., 2007).

3.8 PARASITOLOGY

3.8.1 FAECAL WORM EGG COUNT

For all faecal samples collected from the Pingelly, Arthur River and Frankland properties, the WECs were performed at the State Agricultural Biotechnology Centre (Murdoch University, Western Australia), using a modified McMaster technique (Animal Health Laboratories, 2005c). This method is based on the procedure originally developed by Whitlock (1948) and later described in the Australian Standard Diagnostic Techniques for Animal Diseases Manual (Lyndal-Murphy, 1993). The same technique was utilised for conducting WECs on faecal samples collected from the Boyup Brook and Kojonup properties, although these samples were processed in the Animal Health Laboratories, at the Department of Agriculture and Food, Albany, Western Australia.
The modified McMaster technique described involved weighing exactly 2.0 ± 0.05 g of faeces into a 70mL plastic container. The faeces were allowed to soak in 5mL of water overnight in a refrigerator at 4°C. The faecal sample was mashed using a plastic syringe plunger before adding the floatation salt solution (saturated sodium chloride solution with a specific gravity 1.20 – 1.25) to make the sample suspension up to 60mL. The faecal suspension in the 60mL container was mixed vigorously using a pipette, with 0.6mL taken from the centre of the mixed faecal suspension and added to a counting chamber on a Whitlock Paracytometer Slide. The eggs were counted using the 40x magnification within 45 mins of adding the floatation salt solution. All visible eggs within the external boundaries of the Whitlock Paracytometer counting chamber were recorded. The strongylid and Nematodirus WEC (in epg) were calculated according to Equation 3.2 below:

**Equation 3.2: Determining the WEC epg for faecal samples.**

\[
\text{Number of eggs per gram of faeces} = \frac{\text{number of eggs counted} \times \text{total volume}}{\text{volume of sample counted} \times \text{weight of faeces}}
\]

A Whitlock Paracytometer counting chamber with a volume of 0.6 mL was used resulting in a multiplication factor of 50 for every one strongylid or Nematodirus egg counted. For faecal samples less than 2 g in weight, the following Equation 3.3 was used for correction:

**Equation 3.3: Determining the WEC epg for faecal samples less than 2 g in weight.**

\[
\text{Adjusted faecal weight factor} = \frac{\text{Original multiplication factor} (\times 50 \text{ for Whitlock Paracytometer})}{\text{weight of faecal sample (g)}} \times 2
\]
Strongylidae eggs (often referred to as “strongyle” eggs) and *Nematodirus* spp. eggs were counted separately. Following the counting of worm eggs, coccidia oocysts were searched for in one quarter of a counting chamber to determine if the lamb/sheep was infected with *Eimeria*. If coccidia oocysts were found, the severity of the infection was recorded according to the following measurements; + (low) < 10 oocysts, ++ (moderate) 10–30 oocysts and +++ (high) >30 oocysts. Strongylid and *Nematodirus* worm eggs, along with *Eimeria* (coccidia) oocysts are illustrated in Figure 3.4.

![Figure 3.4: Common sheep WEC parasites eggs and oocysts.](image)

### 3.8.1.1 Factors affecting WEC

Although WECs are a convenient predictor of strongylid worm burden, they are an indirect measurement of strongylid infections. There are several factors that affect WEC and as a result, WEC may not always be highly correlated with the number of established strongylid worms present. These factors have been reviewed by several authors (Kingsbury, 1965; Gordon, 1967; Rubin, 1967; Brunsdon, 1971) and are summarised below:

- The fecundity of female worms is influenced by host factors, which include stress, lactation and immunity. Furthermore, female fecundity may also be influenced by chemotherapy (sub-lethal anthelmintic doses) and nutritional factors.
Faecal worm egg counts are a measure of the concentration of eggs in faeces and therefore WECs are influenced by the volume of faeces produced, movement of digesta through the gastrointestinal tract, as well as the distribution of worm eggs within a faecal mass. Faecal samples from lambs held off feed and with limited water, will as a result have an increased number of strongylid epg, with past studies highlighting the need to adjust WECs for FCS or dry matter percentage (Greeff and Karlsson, 1997; Le Jambre et al., 2007). Therefore all WECs were adjusted for FCS according to the following Equation 3.4 (Le Jambre et al., 2007) below:

**Equation 3.4:** Adjusting WEC for varying faecal consistency score (FCS) (Le Jambre et al., 2007).

\[
\text{Adjusted WEC} = \frac{\text{Raw WEC}}{34.21 - 5.15 \times \text{FCS}} \times 29.06
\]

Worm eggs from different strongylid species are indistinguishable under microscopic analysis and therefore either larval differentiation or PCR is necessary to differentiate *H. contortus*, *Trichostrongylus* spp., *C. ovina*, *Oesophagostomum* spp., *Cooperia* spp. and *T. circumcincta*. This makes clinical interpretation difficult, particularly in animals carrying mixed strongylid species infections.

Eggs are only produced by adult female worms and immature worm burdens or hypobiotic larvae are consequently underestimated.

**3.8.1.2 Interpretation of WEC**

In young sheep (<12 months old), WECs have been proven to be a useful predictor for both strongylid worm burden (correlation *r*=0.74) and seasonal level of infection (correlation *r*=0.99) (Kingsbury, 1965; McKenna, 1981). This is not the case for
Nematodirus WECs, as the correlation between Nematodirus WEC and Nematodirus worm counts is weak \( (r=0.34) \). Hence limited reliance is placed on Nematodirus WECs for diagnostic purposes. This relationship was less evident in adult sheep, although when adult sheep WECs were categorised into “low” (<500 epg), “moderate” (600-2000 epg) or “high” (>2000 epg) (Kingsbury, 1965; McKenna, 1981), a close association was found between WEC and the level of pathogenicity of a mixed strongylid infection for both young and adult sheep. Worm egg count testing was reported to have important diagnostic significance and should be interpreted relative to the history, management and display of any clinical signs within a flock (e.g. diarrhoea, ill thrift, anaemia and anthelmintic treatment history) (Woodgate and Besier, 2010).

3.8.2 LARVAL CULTURE DIFFERENTIATION

Larval cultures were conducted to differentiate between those strongylid species present in worm eggs. These cultures were performed at the Animal Health Laboratories, Department of Agriculture and Food, Albany, Western Australia (Animal Health Laboratories, 2005a). The techniques utilised for larval cultures and also for the identification of strongylid species were based on diagnostic methods described in the Australian Standard Diagnostic Techniques for Animal Disease Manual (Lyndal-Murphy 1993). Equal amounts (3–5 g) from each sample were placed in a culture bottle to make a 30 g sample and combined with 20 mL water before mixing with 5 g of medium grade vermiculite to give a crumbly mixture. A lid was place on the bottle and it was then incubated at 27°C for seven days. After incubation, the culture was exposed to light for 1 hr, after which the culture bottle was filled with water (30°C), inverted in a glass Petri dish and the surrounding ‘moat’ filled with water. The culture bottle was left inverted for 3-8 hr to allow larvae to collect in the surrounding moat. The liquid and larvae were then pipetted
into a centrifuge tube and allowed to settle out before removing some of the supernatant carefully with a pipette. Parasitological iodine was added to kill the larvae which were then identified under light microscopy. One hundred larvae (or as many as seen if less than 100) were differentiated into species and the results expressed as a percentage (%) for each species.

3.8.3 PASTURE LARVAL COUNTS

Pasture from the paddock(s) grazed by sheep on the Frankland property was collected from 200 sites at roughly equal distances along a W-shaped transect (Taylor, 1939; Martin et al., 1990). Four “plucks” of pasture were collected at each site from in front, behind, left and right of the sampler. Where possible, “plucks” did not include roots, soil or faeces. Pasture was kept at 2–4°C until processed.

Pasture larval counts were conducted by Animal Health Laboratories, Department of Agriculture and Food (Albany, Western Australia) using the method described in the Animal Health Laboratory Research Methods (Animal Health Laboratories, 2005b) and modified according to Martin et al. (1990). This technique detects all three strongylid nematode larval stages, however it is difficult to differentiate L₁ and L₂ stages from other free-living non-parasitic nematodes.

Briefly, larvae were washed off the pasture and allowed to settle in large bins. The sediment was collected and concentrated using further sedimentation steps until a volume of at least one part sediment to one part liquid was obtained. This was followed by a number of floatation and centrifugation steps utilising a saturated potassium iodide solution (K₁:water, 1:1) as a floatation medium. The supernatant was filtered to remove the potassium iodide and resuspended in water. Following further centrifugation and re-
suspension of the sediment in a saturated potassium iodide solution, a 10% aliquot of the sample was examined using microscopy (Martin et al., 1990; Animal Health Laboratories, 2005b).

3.9 DNA EXTRACTION, AMPLIFICATION AND SEQUENCING

3.9.1 EXTRACTION OF GENOMIC DNA FROM FAECES

Genomic DNA was extracted directly from 250–300 mg crude faecal samples using a Power Soil DNA Kit (MolBio, West Carlsbad, California, USA) (Yang et al., 2009; Yang et al., 2011). Modifications to the manufactures protocol are included in bold text in Appendix 13.2.

A negative control (known parasite negative faecal sample) and positive control (faecal sample spiked with Cryptosporidium and Giardia [oo]cysts or strongylid L₃) were used in each extraction group. Eluted DNA was stored at −20°C until required.

3.9.2 EXTRACTION OF GENOMIC DNA FROM STRONGYLID LARVAE

Genomic DNA was extracted from strongylid L₃ and used for positive controls in the strongylid species specific PCR assays. Five species of L₃ (T. colubriformis, T. circumcincta, H. contortus, Oesophagostomum spp. and C. ovina) were identified and separated into 1.5 mL Eppendorf tubes. DNA tissue extraction from ~200 µL of the L₃ suspension were performed using the DNeasy® Blood and Tissue Kit (Qiagen) following the manufacturer's protocol (purification of total DNA from Animal Tissues, Qiagen). Briefly:

1. Pre-heat heat Buffer ATL and Buffer AL to remove any form of possible precipitation.
2. Add 180 µL of Buffer ATL to each 200 µL suspension of strongylid L₃.
3. Add 20 µL proteinase K, mix thoroughly by vortexing and incubating at 56°C until the tissue is completely lysed (vortex occasionally during incubation to disperse the sample or place the sample in a shaking water bath). Lysis varies depending on the tissue type processed, with samples often lysed overnight (performed in this study).

4. Vortex for 15 s, then add 200 µL of Buffer AL to each sample and mix thoroughly by vortexing. Then add an extra 200 µL ethanol (96–100%) and mix again thoroughly by vortexing.

5. Pipette the mixture from step 4 (including precipitate) into a DNeasy Mini column and place in a 2 mL collection tube. Centrifuge at ≥6000 x g (8000 rpm) for 1 min. Discard flow through and collection tube.

6. Place the DNeasy Mini spin column in a new 2 mL collection tube, add 500 µL of Buffer AW1 and centrifuge at ≥6000 x g (8000 rpm) for 1 min. Discard flow through and collection tube.

7. Place the DNeasy Mini spin column in a new 2 mL collection tube, add 500 µL of Buffer AW2 and centrifuge at 20000 x g (14000 rpm) for 3 mins to dry DNeasy membrane. Discard flow through and collection tube.

8. Place the DNeasy Mini spin column in a new 1.5–2 mL microcentrifuge tube, and pipette 100 µL of Buffer AE directly onto DNeasy membrane. Incubate at room temperature for 1 min and then centrifuge at ≥6000 x g (8000 rpm) for 1 min to elute.

9. For maximum DNA yield, repeat elution step described in step 8.

Purified DNA was stored at −20°C, until used as positive controls for strongylid worm PCRs.
3.9.3 EXTRACTION OF GENOMIC DNA FROM LIVESTOCK WATER

A 10 L water sample was collected from each of the relevant livestock water sources on the Pingelly, Arthur River and Frankland properties. Pingelly lambs drank water from a flowing creek along which water soakings were constructed to trap water, while Arthur River and Frankland lambs drank from clay based dams or from a trough sourcing water from clay based dams. These water samples were transported to Murdoch University State Animal Biotechnology Laboratory and stored at 4°C until tested within 48 hr. Using Envirochek filters (Pall Life Sciences, New South Wales, Australia), capsule filtration was performed in accordance with the manufacturer’s instructions, except that the Laureth 12 elution buffer was replaced with a phosphate-buffered saline (PBS) Tween-Antifoam buffer (2L of PBS buffer [pH 7.4], 300 µL of Tween 80, and 300 µL of Antifoam B). This change to manufacturer’s instructions was based on demonstrated improvements in percent recoveries in previous trials and on the recommendation of Hunter Water Laboratories, New South Wales, Australia. One hundred millilitres of PBS elution buffer was added to the capsule and placed in a wrist action shaker for 5 min with the bleed valve set at the 12 o’clock position. Eluate was collected into a 200 mL centrifuge tube. The elution procedure was repeated once more, changing the orientation of the filter in the shaker to either the 3 o’clock or 9 o’clock position. The second wash eluate was combined in the same tube and centrifuged at 1,100 x g for 15 min. The supernatant was aspirated, and DYNAL immunomagnetic separation was performed on the pellet Wohlsen et al. (2004). Following this separation for purification of any oocysts or cysts, the final complex was subjected to the same genomic DNA extraction method as were the faecal samples and is detailed above.
3.9.4 DNA AMPLIFICATION

3.9.4.1 Amplification of Cryptosporidium at the 18S rRNA gene

A two-step nested PCR was used to amplify the 18S rRNA gene for Cryptosporidium, with the primary amplified product of ~763 base pairs (bp) using the forward primer 18SiCF2 (5’ – GAC ATA TCA TTC AAG TTT CTG ACC – 3’) and the reverse primer 18SiCR2 (5’ – CTG AAG GAG TAA GGA ACA ACC – 3’) (Ryan et al., 2003). The PCR mixture consisted of 400 mM (each) deoxynucleotide triphosphates (dNTP) (Fisher Biotech, Perth, Australia), 1 x kapa Taq DNA polymerase buffer (Kapa Biosystems, Cape Town, South Africa), 2.5 mM MgCl₂ (Fisher Biotech), 0.05U/µL of kapa Taq DNA Polymerase (Kapa Biosystems, Cape Town, South Africa) and 0.80 µM of forward and reverse primers. PCR reactions containing 1 µL of DNA were amplified in a total volume of 25 µL. Following a 5 min preliminary heating at 95°C for 5 mins, 50 PCR cycles (95°C for 30 s, 58°C for 30 s, 72°C for 60 s) were carried out in an Applied Biosystems Gene Amp PCR System 2700 thermocycler, finished with a 7 min extension at 72°C. A positive control of Cryptosporidium hominis was used and a negative control (no DNA added) was included for all sets of reactions.

For the secondary PCR, a product of ~580bp was amplified using 1µL of primary PCR product and nested forward 18SiCF1 (5’ – CCT ATC AGC TTT AGA CGG TAG G – 3’) and reverse 18SiCR1 (5’ – TCT AAG AAT TTC ACC TCT GAC TG – 3’) primers (Ryan et al., 2003). The conditions for the secondary PCR were identical to the primary. Positive secondary PCR products were sequenced directly in the reverse direction.
3.9.4.2 Amplification of Cryptosporidium at the actin gene

All Cryptosporidium positive samples at the 18S rRNA locus were re-screened by a nested PCR amplification of the actin gene. A primary PCR product of 868bp was amplified using the forward actin AII F1 (5’ –ATG CCV GGW RTW ATG GTD GGT ATG – 3’) and the reverse primer actin Act6R (5’ –GGD GCA ACR ACY TTR ATC TTC – 3’) in the primary PCR (Ng et al., 2006). The PCR mixture consisted of 400 mM (each) deoxynucleotide triphosphates (dNTP) (Fisher Biotech, Perth, Australia), 1 x kapa Taq DNA polymerase buffer (Kapa Biosystems, Cape Town, South Africa), 2.5 mM MgCl₂ (Fisher Biotech), 0.05U/µL of kapa Taq DNA Polymerase (Kapa Biosystems, Cape Town, South Africa) and 0.80 µM of forward and reverse primers. PCR reactions containing 1 µL of DNA were amplified in a total volume of 25 µL. Following a 5 min preliminary heating at 95°C for 5 mins, 50 PCR cycles (95°C for 30 s, 58°C for 30 s, 72°C for 60 s) were carried out in an Applied Biosystems Gene Amp PCR System 2700 thermocycler, then finished with a 7 min final extension at 72°C. A positive control and negative control (no DNA added) were included for all sets of reactions.

In the secondary PCR, a fragment of ~818bp was amplified using 1 µL of primary PCR product and forward actin AII F2 (5’ – GAY GAR GCH CAR TCV AAR AGR GGT AT – 3’) and reverse actin AII R1 (5’ – TTD ATY TTC ATD GTH GAH GGW GC – 3’) primers (Ng et al., 2006). The conditions for the secondary PCR were identical to the primary PCR. Positive secondary PCR products were sequenced directly in the reverse direction.

3.9.4.3 Amplification of C. parvum at a diagnostic locus by qPCR

All samples positive for Cryptosporidium mixed infections (C. xiaoi and C. parvum, C. ubiquitum and C. parvum) were further screened using a C. parvum and C. hominis
qPCR at a unique Cryptosporidium specific protein coding locus previously described by Yang et al., (2009). This Cryptosporidium-specific protein-coding (Morgan-Ryan et al., 1997) is subsequently referred to in this paper as the diagnostic locus. Briefly, a forward primer, 021F (5’ – GGT ACT GGA TAG ATA GTG GA – 3’), which anneals to both C. hominis and C. parvum was combined in the same reaction with a C. hominis-specific primer, CHR (5’ – CCT CTT TCC AAT TAA AGT TGA TG – 3’) and a C. parvum-specific primer CPR (5’ – TCC AAA TTA TTG TAA CCT GGA AG – 3’). A C. hominis-specific probe (5’ – FAM-TGA TTT TCC AGG CTA C – 3’) and C. parvum-specific probe (5’ – JOE-TGA TCT TCC AGG TTA C – 3’) were also included. Each 15 mL PCR mixture contained 1 µL HotStar kapa Taq Buffer (Kapa Biosystems, Cape Town, South Africa), 5 mM MgCl₂, 1mM dNTPs, 0.05U/µL Hot Start kapa Taq DNA polymerase (Kapa Biosystems, Cape Town, South Africa), 0.2 mM each of forward and reverse primers and 50 nM each of the C. hominis and C. parvum probes. The PCR cycling conditions consisted of a pre-melt at 95°C for 10 min, followed by 45 cycles of 95°C for 30 s and then a combined annealing and extension step of 61°C for 60 s. A standard curve for quantifying C. parvum/C. hominis DNA was generated using DNA extracted from 10,000 C. parvum and/or C. hominis oocysts and diluted down to 1000, 100, 10 and 1 oocyst equivalents.

A spike analysis (addition of 0.5 mL of C. hominis) was conducted on randomly selected Cryptosporidium negative samples from each group of DNA extractions to determine if negative results were caused by PCR inhibition.

3.9.4.4 Amplification of C. parvum at the gp60 gene

All C. parvum positive samples were subtyped by a nested PCR amplification at the 60–kDA glycoprotein (gp60) locus. A primary PCR product of ~1000bp was amplified using
the forward \( gp60 \) F1 (5’ – ATA GTC TCC GCT GTA TTC – 3’) and the reverse primer \( gp60 \) R1 (5’ – TCC GCT GTA TTC TCA GCC – 3’) in the primary PCR (Strong et al., 2000; Glaberman et al., 2002; Sulaiman et al., 2005). The PCR mixture consisted of 400 mM (each) deoxynucleotide triphosphates (dNTP) (Fisher Biotech, Perth, Australia), 1 x DNA polymerase buffer (Fisher Biotech, Perth, Australia), 3 mM MgCl\(_2\) (Fisher Biotech, Perth, Australia), 0.05 U/µL of \( tth^+ \) Taq DNA Polymerase (Fisher Biotech, Perth, Australia) and 0.80 µM of forward and reverse primers. PCR reactions containing 1 µL of DNA were amplified in a total volume of 25 µL. Following a 5 min preliminary heating at 95°C for 5 mins, 35 PCR cycles (95°C for 45 s, 58°C for 45 s, 72°C for 60 s) were carried out in an Applied Biosystems Gene Amp PCR System 2700 thermocycler, then finished with a 10 min final extension at 72°C. A positive control and negative control (no DNA added) were included for all sets of reactions.

In the secondary PCR, a fragment of ~850bp was amplified using 2 µL of primary PCR product and forward \( gp60 \) F2 (5’ – GGA AGG AAC GAT GTA TCT – 3’) and reverse \( gp60 \) R2 (5’ – GCA GAG GAA CCA GCA TC – 3’) primers (Strong et al., 2000; Glaberman et al., 2002; Sulaiman et al., 2005). The conditions for the secondary PCR were identical to the primary PCR. Positive secondary PCR products were sequenced directly in the reverse direction.

### 3.9.4.5 Amplification of *Giardia* at the glutamine dehydrogenase gene

A semi-nested PCR was used to screen for *Giardia* at the \( gdh \) (glutamate dehydrogenase) gene, with the primary amplified a product of ~480bp using the external forward primer GDHeF (5’ – TCA ACG TYA AYC GYG GYT TCC GT – 3’), internal forward primer GDHiF (5’ – CAG TAC AAC TCY GCT CTC GG – 3’) and the reverse primer GDHiR
(5’ – GTT RTC CTT GCA CAT CTC C – 3’) (Read et al., 2004). Primers contained degenerate bases (“Y”) to enable amplification of isolates across all assemblages. The PCR mixture consisted of 400 mM (each) deoxynucleotide triphosphates (dNTP) (Fisher Biotech, Perth, Australia), 1 x kapa Taq DNA polymerase buffer (Kapa Biosystems, Cape Town, South Africa), 2.5 mM MgCl₂ (Fisher Biotech, Perth, Australia), 0.05U/µL of kapa Taq DNA Polymerase (Kapa Biosystems, Cape Town, South Africa) and 0.80 µM of forward and reverse primers. PCR reactions containing 1 µL of DNA were amplified in a total volume of 25 µL. Following a 5 min preliminary heating at 95°C for 5 mins, 50 PCR cycles (95°C for 30 s, 56°C for 30 s, 72°C for 50 s) were carried out in an Applied Biosystems Gene Amp PCR System 2700 thermocycler, then finished with a 7 min extension at 72°C. A positive control of Giardia duodenalis assemblage A was used and a negative control (no DNA added) was included for all sets of reactions.

3.9.4.6 Amplification of Giardia at the triosephosphate isomerase gene

All Giardia positives isolated at the gdh gene were screened using an assemblage specific amplification nested PCR at the tpi (triosephosphate isomerase) gene. The primary PCR, a PCR product of ~605bp was amplified using external forward primer AL3543 (5’ – AAA TIA TGC CTG CTC GTC G – 3’) and the reverse primer AL3546 (5’ – GTT RTC CTT GCA CAT CTC C – 3’) (Sulaiman et al., 2003). The PCR mixture consisted of 400 mM (each) deoxynucleotide triphosphates (dNTP) (Fisher Biotech, Perth, Australia), 1 x DNA polymerase buffer (Fisher Biotech, Perth, Australia), 2.5 mM MgCl₂ (Fisher Biotech, Perth, Australia), 0.05U/µL of tth+ Taq DNA Polymerase (Fisher Biotech, Perth, Australia) and 0.80 µM of forward and reverse primers. PCR reactions containing 2.5 µL of DNA were amplified in a total volume of 25 µL. Following a 5 min preliminary heating at 94°C, 40 PCR cycles (94°C for 45 s, 56°C for 45 s, 72°C for 60 s) were carried out in an Applied Biosystems Gene Amp PCR System 2700 thermocycler, then finished with a 7 min extension at 72°C. A positive control of Giardia duodenalis assemblage A was used and a negative control (no DNA added) was included for all sets of reactions.
Biosystems Gene Amp PCR System 2700 thermocycler, then finished with a 10 min extension at 72°C.

Each *Giardia* positive, was screened with two assemblage specific secondary PCRs, using a 1 in 10 dilution of the first round PCR as a template. The first PCR assay was for assemblage A, with assemblage A specific forward primer Af (5’ – CGC CGT ACA CCT GTC A – 3’) and reverse primer (5’ – AGC AAT GAC AAC CTC CTT CC – 3’), amplifying ~332bp PCR product (Geurden *et al.*, 2008a; Geurden *et al.*, 2009). The second assemblage-specific PCR screened for assemblage E, with specific forward Ef (5’ – CCC CTT CTG CCG TAC ATT TAT – 3’) and reverse Er (5’ - GGC TCG TAA GCA ATA ACG ACT T – 3’) amplifying a ~388bp PCR product (Geurden *et al.*, 2008a; Geurden *et al.*, 2009). The reaction mixture for the secondary assemblage-specific tpi PCR consisted of 400 mM (each) deoxynucleotide triphosphates (dNTP) (Fisher Biotech, Perth, Australia), BSA at a final concentration of 0.1 µg/µL 1 x DNA polymerase buffer (Fisher Biotech, Perth, Australia), 2.5 mM MgCl₂ (Fisher Biotech, Perth, Australia), 0.05U/µL of *tth+* Taq DNA Polymerase (Fisher Biotech, Perth, Australia) and 0.80 µM of forward and reverse primers. PCR reactions containing 2.5µL of diluted template DNA were amplified in a total volume of 25 µL. Following a 10 min preliminary heating at 94°C, 40 PCR cycles (94°C for 45 s, 64°C for 45 s for assemblage A specific primers or 67°C for assemblage E specific primers, 72°C for 60 s) were carried out in an Applied Biosystems Gene Amp PCR System 2700 thermocycler, finished with a 10 min extension at 72°C. A positive control of *G. duodenalis* assemblage A was used and a negative control (no DNA added) was included for all sets of reactions.
3.9.4.7 Amplification of *Giardia* at the β–giardin gene

A semi-nested PCR was used to screen for *Giardia gdh* positives at the β–giardin gene, with the primary amplified a product of ~753bp using the forward G7 (5’ – AAG CCC GAC GAC CTC ACC CGC AGT GC – 3’) and reverse G759 (5’ – GAG GCC GCC CTG GAT CTT CGA GAC GAC – 3’) primers (Cacciò *et al.*, 2002). These primers were designed using the complete β–giardin sequence of the Portland-1 isolate (GenBank accession number M36728). The PCR mixture consisted of 400mM (each) deoxynucleotide triphosphates (dNTP) (Fisher Biotech, Perth, Australia), 1 x *kapa* Taq DNA polymerase buffer (Kapa Biosystems, Cape Town, South Africa), 2.5 mM MgCl$_2$ (Fisher Biotech, Perth, Australia), 0.05U/µL of *kapa* Taq DNA Polymerase (Kapa Biosystems, Cape Town, South Africa) and 0.80 µM of both forward and reverse primers. PCR reactions containing 1 µL of DNA were amplified in a total volume of 25 µL. Following a 5 min preliminary heating at 95°C for 5 mins, 50 PCR cycles (95°C for 30 s, 65°C for 30 s, 72°C for 60 s) were carried out in an Applied Biosystems Gene Amp PCR System 2700 thermocycler, then finished with a 7 min extension at 72°C. A positive control of *Giardia duodenalis* assemblage A was used and a negative control (no DNA added) was included for all sets of reactions.

In the secondary PCR, a fragment of ~384bp was amplified using 1 µL of primary PCR product and forward G376 (5’ – GGA AGG AAC GAT GTA TCT – 3’) and reverse G759 (Cacciò *et al.*, 2002). The conditions for the secondary PCR were identical to those of the primary PCR, except that an annealing temperature of 55°C was used, instead of 65°C. Positive secondary PCR products were sequenced directly in the reverse direction.
3.9.4.8 Amplification of strongyloid species at the ITS-2 rDNA locus together with 28S rRNA gene

Species-specific PCR was performed to detect each of the following strongyloid species (T. circumcincta, Trichostrongylus spp., H. contortus, Oesophagostomum spp. and C. ovina). Individual forward primers (TEL, TRI, HAE and CHO) designed for the second internal transcribed spacer (ITS-2) of ribosomal DNA and the reverse primer (NC2) located at the 5’-region of the 28S rRNA gene, were used to detect genomic DNA from strongyloid worm eggs or larvae (Bott et al., 2009). The PCR reported a high sensitivity following amplification of DNA extracted from eggs, following sodium nitrate and column-purification from faeces; with a minimum detection limit ranging between of 0.1 – 2pg of genomic DNA (Bott et al., 2009). Primer pairs for each strongyloid species include: TEL–NC2 (T. circumcincta), TRI–NC2 (Trichostrongylus spp. and Oesophagostomum spp.), HAE–NC2 (H. contortus) and CHO – NC2 (C. ovina). The primer pair TRI–NC2 has been shown to amplify all four major Trichostrongylus species if established as patent infections. These included Trichostrongylus colubriformis, T. axei, T. vitrinus and T. rugatus (Bott et al., 2009). The TRI–NC2 primer pair also amplified both Oesophagostomum species (Oesophagostomum columbianum and Oesophagostomum venulosum), represented by a fainter 160 bp product (Bott et al., 2009). Primer characteristics are detailed in Table 3.3.
Table 3.3: Specific forward primers used with the reverse primer (NC2) to detect patent strongylid infections by PCR.

<table>
<thead>
<tr>
<th>Species</th>
<th>Primer</th>
<th>Primer sequence</th>
<th>GC (%)</th>
<th>Melting temperature °C</th>
<th>Amplicon product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemonchus contortus</td>
<td>HAE</td>
<td>CAAATGGCAATTGTCTTTTAG</td>
<td>33</td>
<td>47</td>
<td>265</td>
</tr>
<tr>
<td>Teladorsagia circumcincta</td>
<td>TEL</td>
<td>TATGCAACATGACGTACGCGG</td>
<td>50</td>
<td>55</td>
<td>218</td>
</tr>
<tr>
<td>Trichostrongylus spp.</td>
<td>TRI</td>
<td>TCGAATGGTCATTGCAA</td>
<td>37</td>
<td>45</td>
<td>267-268</td>
</tr>
<tr>
<td>Oesophagostomum spp.</td>
<td>TRI</td>
<td>TCGAATGGTCATTGCAA</td>
<td>37</td>
<td>45</td>
<td>160</td>
</tr>
<tr>
<td>Oesophagostomum venulosum</td>
<td>OEV</td>
<td>TGAAATGAGACAACCGTAGTCG</td>
<td>45</td>
<td>53</td>
<td>105</td>
</tr>
<tr>
<td>Chabertia ovina</td>
<td>CHO</td>
<td>GATGACCTCGTTGTCACCGTG</td>
<td>57</td>
<td>56</td>
<td>162</td>
</tr>
</tbody>
</table>

The PCR reactions were performed using 1 μL of DNA in a 25 μL reaction containing 1 x *kapa* Taq DNA polymerase buffer (Kapa Biosystems, Cape Town, South Africa), 2.5 mM MgCl₂ (Fisher Biotech, Perth, Australia), 0.4 mM dNTPs (Fisher Biotech, Perth, Australia), 0.80 μM of each primer and 0.5U/μL of *kapa* Taq DNA Polymerase (Kapa Biosystems, Cape Town, South Africa). Following a 5 min preliminary heating at 94°C for 5 mins, 40 PCR cycles (94°C for 30 s, 55°C for 30 s, 72°C for 45 s) were carried out in an Applied Biosystems Gene Amp PCR System 2700 thermocycler, then finished with a 7 min final extension at 72°C. A positive control and negative control (no DNA added) were included for all sets of reactions to detect possible contamination.

3.9.4.9 Amplification of strongylid species at the ITS-2 locus by qPCR

Species-specific qPCR assays were performed to detect each of the following strongylid species (*T. circumcincta*, *Trichostrongylus* spp., *H. contortus*, *Oesophagostomum venulosum* and *C. ovina*). Individual forward primers (TEL, TRI, HAE CHO and OEV) designed for the second internal transcribed spacer (ITS-2) of ribosomal
DNA and the reverse primer (NC2) located at the 5′-region of the 28S rRNA gene, were used to detect genomic DNA from strongylid worm eggs or larvae (Bott et al., 2009). Primer pairs for each strongylid species include: TEL–NC2 (T. circumcincta), TRI–NC2 (Trichostrongylus spp.), HAE–NC2 (H. contortus), CHO–NC2 (C. ovin) and OEV–NC2 (Oesophagostomum venulosum). The primer pair TRI–NC2 has been shown to amplify all four major Trichostrongylus species if established as patent infections, which included T. colubriformis, T. axei, T. vitrinus and T. rugatus (Bott et al., 2009). The qPCR assays were conducted according to Bott et al., (2009), while PCR assays procedures are described above and in Table 3.3.

The Cq values (fractional cycle number at the point where the amplification curve crosses a threshold of detection) for each sample were established by setting threshold lines and calculating the intersection with each of the sample curves. Samples that crossed the threshold before 40 cycles were classified as positive and any samples that didn’t cross the threshold after 40 cycles were classified as negative (Bustin et al., 2009; Taylor et al., 2010).

3.9.4.10 Amplification of Salmonella and C. jejuni by PCR

All samples Salmonella positive by qPCR were re-screened at the ompF locus. A single step PCR protocol was used to amplify ompF locus of Salmonella as previously described by Tatavarthy and Cannons (2010), producing a product of ~578bp. All samples C. jejuni positive by qPCR were re-screened at the 16S rRNA locus. A single step PCR protocol was used to amplify 16S rRNA locus of C. jejuni as previously described by Lubeck et al., (2003), producing a product of ~287bp.
A spike analysis (addition of 0.5 mL of *S. enteric* or *C. jejuni*) was conducted on randomly selected *Salmonella* and *C. jejuni* negative samples from each group of DNA extractions to determine if negative results were caused by PCR inhibition.

### 3.9.5 AGAROSE GEL ELECTROPHORESIS

Horizontal electrophoresis was performed using 1.0% agarose gels (Promega, Madison, USA) in 1x TAE buffer (40 mM Tris-HCL; 20mM acetate; 2mM EDTA pH adjusted to 8.0) stained with x 0.025µL/mL SYBER® safe DNA gel stain (Invotrgoen, Carlsbad, USA). Post electrophoresis PCR product visualisation was performed by UV transillumination using a BIO Rad Gel Doc 1000 transilluminator.

### 3.9.6 PRE SEQUENCE REACTION DNA PURIFICATION FROM GEL

All secondary PCR positive products were purified using an UltraClean™ DNA Purification Kit (MolBio, West Carlsbad, California, USA) and sequenced using an ABI Prism™ Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) on an Applied Biosystem 3730 DNA Analyzer. The procedure for purifying DNA from gel bands was conducted as described below:

1. Determine volume of DNA in solution
2. Add 3 volumes of ULTRA SALT to 1.5 mL Eppendorf tube, i.e. gel weight = 0.1 g, add 300 mL of ULTRA SALT.
3. Mix well
4. Incubate at 55°C to melt agarose gel. Mix occasionally by shaking thoroughly. Completely melt gel band before proceeding (~5 mins).
5. Resuspend ULTRA BIND by vortexing the ULTRA BIND tube at the highest speed in a horizontal position until homogeneous (~1 mins).
6. Add 6 µL of ULTRA BIND to each 1.5 mL Eppendorf tube to recover DNA.
7. Incubate for 10 mins at room temperature, while incubating vortex each tube several times.
8. Centrifuge at 20,000 x g for 5 s. Remove the supernatant.
9. Resuspend the pellet in 1 mL of ULTRA WASH by vortexing for 30 s.
10. Centrifuge at 20,000 x g for 5 s. Remove the supernatant.
11. Place in rotary air vacuum for ~15 mins to remove all traces of ULTRA WASH.
12. Add 20 µL of PCR PURE WATER to each pellet and resuspend.
13. Heat at 55°C for 5 mins.
14. Centrifuge each sample at 20,000 x g for 1 min.
15. Transfer supernatant into a clean 1.5 mL Eppendorf tube. DNA is now ready for use.

3.9.7 SEQUENCE REACTION

Purified secondary PCR products were sequenced using a Big Dye version 3.1 Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California) according to the manufacturer’s instructions. One eighth reactions were performed with the reaction mixture containing 1 µL of 5x sequencing buffer (Applied Biosystems), 6.25 pmoles primer and 20 ng DNA template for Cryptosporidium, Giardia or strongylid purified PCR product made up to a final volume of 10 µL with PCR grade water (Fisher Biotech). Thermal cycling conditions for the sequence reaction included an initial hold on 96°C for 2 mins, followed by 35 cycles of 96°C for 10 s, 58°C for 5 s and 60°C for 4 mins.
3.9.8 POST REACTION PURIFICATION USING ETHANOL PRECIPITATION

Following completion of the sequence reaction, a 0.5 mL Eppendorf tube was prepared in the following order for DNA purification:

1. 1 µL of 125 mM Disodium salt (EDTA) added to an empty 0.5 mL Eppendorf tube.
2. The full 10 µL sequencing reaction was added to the 0.5 mL Eppendorf tube.
3. 1 µL of 3M sodium acetate pH 5.2 was added to the 0.5 mL Eppendorf tube.
4. Finally 25 µL of 100% ethanol was added, with the contents in the Eppendorf tube mixed by pipetting and then left to stand at room temperature for 20 mins.

The samples were then centrifuged at 20,000 x g for 30 mins and the supernatant discarded. The DNA pellet was then washed with 125 µL 70% ethanol, and centrifuged at 20,000 x g for 5 mins and the supernatant discarded. The pellet was then subjected to a final 25 µL 70% ethanol and centrifuged at 20,000 x g for 5 mins.

3.10 SEQUENCE AND PHYLOGENETIC ANALYSIS

Sequence searches of positive isolates for Cryptosporidium, Giardia and strongylids were conducted using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and nucleotide sequences were analysed using Chromas Lite version 2.0 (http://www.technelysium.com.au) and aligned with reference genotypes from GenBank using Clustal W (http://www.clustalw.genome.jp). Phylogenetic trees were constructed for Cryptosporidium isolates at 18S rRNA and actin loci and Giardia isolates at the gdh and β-
giardin loci, with additional isolates obtained from GenBank. Distance estimation was conducted firstly using TREECON for Windows (Van de Peer and De Wachter, 1994), based on evolutionary distances between isolates calculated by Kimura 2-parameter model and grouped using Neighbour-Joining and secondly using Mega 5 software to conduct maximum-parsimony (Kumar et al., 2008). The confidence of groupings was assessed by bootstrapping, using 1000 replicates. A percentage bootstrap support of >50% was used for each phylogenetic tree.

3.11 STATISTICS

Statistical analyses were performed using SPSS Statistics 17.0 (Statistical Package for the Social Sciences) for Windows (SPSS inc. Chicago, USA). The prevalence (including 95% confidence intervals) at each sampling occasion and overall (lambs positive for the respective parasite on either first or second sampling occasion) were calculated using the exact binomial method (Thrusfield, 2007).

The WECs were adjusted for FCS to estimate WEC that would be expected if the samples were FCS=1 (consistency of normal faeces) according to Equation 3.4.

Adjusted WEC data were assessed for normality of data distribution and homogeneity of variance. The WEC data were transformed using $\log_{10}(\text{adjusted WEC}+25)$ to stabilise variances between groups prior to statistical analysis (Dobson et al., 2009).

Greater details of the individual statistical analyses conducted are provided in each of the following research Chapters.
3.12 CONFLICT OF INTEREST STATEMENT

The researcher and supervisory team had no financial or personal relationships with other people or organisations that could inappropriately influence or bias the content of this research in this thesis.
CHAPTER 3: MATERIALS AND METHODS

CHAPTER 4: SURVEY OF DIARRHOEA IN MEAT LAMB FLOCKS IN WA

PREVALENCE AND ON-FARM RISK FACTORS FOR DIARRHOEA IN MEAT LAMB FLOCKS IN WA


4.1 INTRODUCTION

Diarrhoea is a significant economic and welfare problem for sheep enterprises worldwide (Larsen et al., 1999; Sargison, 2004; Jacobson et al., 2009a). It poses a major risk factor for the accumulation of faeces on fleece at the breech (perineal region) and for outbreaks of cutaneous myiasis (‘blowfly strike’) (Morley et al., 1976; French et al., 1994; Hall and Wall, 1995; Snoep et al., 2002; Bisdorff and Wall, 2008). Furthermore, breech fleece faecal soiling increases the risk of carcase contamination with enteric microbes associated with meat spoilage and human food poisoning (Greer et al., 1983; Hadley et al., 1997). In addition, faecal contamination of carcases necessitates trimming of effected carcase tissues, that in turn limits abattoir productivity (Hadley et al., 1997).

Despite the widespread nature of diarrhoea in sheep enterprises and the serious economic and animal welfare consequences, little information on diarrhoea prevalence and potential farm management risk factors have been reported. A large number of infectious and non-infectious agents have been associated with diarrhoea in naive lambs, including strongylid nematodes (Taylor et al., 1993; Eerens et al., 1998; Sargison, 2004), protozoan
(Cryptosporidium, Giardia) and coccidian (Eimeria) parasites (Olson et al., 1995; Causapé et al., 2002; Aloisio et al., 2006). Apart from diarrhoea, strongylid nematodes and protozoa adversely affect lamb meat productivity, through reduced growth rates and subsequent carcase weights of infected lambs (Sackett et al., 2006; Sutherland et al., 2010). Further information regarding the pathogenicity of internal parasites in sheep is presented in the Literature Review (Chapter Two).

The WA sheep industry is changing, with an overall decline in the state’s total number of sheep and enterprises increasing the focus of their production towards the meat market (Fletcher et al., 2009; ABARE, 2010). Control of strongylid nematodes is a major challenge for sheep enterprises, due to widespread anthelmintic resistance, particularly in Western Australia where resistance to several anthelmintic treatment groups has been reported (Palmer et al., 2001; Besier and Love, 2003; Suter et al., 2005). Sheep producers commonly administer anthelmintic treatments to flocks following diarrhoea outbreaks and an increased anthelmintic treatment frequency has been linked with an increased risk in the development of anthelmintic resistance in worm populations (Besier and Love, 2003; Woodgate and Besier, 2010).

4.1 AIMS AND HYPOTHESES

The aims of this study utilising a questionnaire survey were to:

1. Investigate the reported prevalence of diarrhoea outbreaks in meat lamb enterprises in WA.
2. Determine the observed proportion of meat lamb flocks effected with diarrhoea.
3. Report sheep management practices relevant to conditions associated with diarrhoea (including strongylid nematode control).

4. Identify risk factors for diarrhoea outbreaks.

5. Examine producer awareness of internal parasites.

6. Assess producer responses to different, defined diarrhoea scenarios.

The hypotheses of this questionnaires survey were that:

1. Diarrhoea outbreaks are common and widespread through all agricultural zones.

2. Diarrhoea outbreaks are most prevalent in areas of high annual rainfall.

3. Anthelmintic treatment is a significant risk factor for diarrhoea outbreaks.

4. The majority of producers are unaware of Cryptosporidium and Giardia infecting sheep.

5. Producers frequently utilise WECs to determine if an anthelmintic treatment is warranted for a lamb flock.

4.2 MATERIALS AND METHODS

4.2.1 STUDY POPULATION AND MAILING

In a recent financial performance report of the sheep industry in Australia, 7,100 farms in Western Australia were reported to run a sheep enterprise (wool or meat production) with a reported 14.7 million sheep at the end of 2010. Of these, a total of 1,316 farms were reported to have a specialised lamb meat enterprise that sent lambs for slaughter to commercial abattoirs (ABARE, 2010; Athas, 2011). A total of 139 lamb meat
enterprises responded to the questionnaire in this current study, accounting for 10.6% of Western Australia’s specialised lamb meat farms.

4.2.2 QUESTIONNAIRE SURVEY DESIGN

This research conforms to the international reporting guidelines of strengthening the reporting of observational studies in epidemiology (STROBE) (Vandenbroucke et al., 2007; von Elm et al., 2007) and was approved by the Murdoch University Human Research Committee approved the questionnaire (HREC permit number 2009/222).

The questionnaire consisted of a cover note which explained the aims of the study, followed by 20 questions relating to the prevalence of diarrhoea observed in meat (slaughter) lambs during 2010, sheep management, internal parasite control and awareness and response to different diarrhoea outbreak scenarios. Questions included; time of lambing, whether respondents observed diarrhoea in meat lambs, the proportion of lambs affected, the month diarrhoea was first observed, administration of anthelmintics to pregnant ewes (before lambing) or lambs, presence of cattle on the property, utilisation of WECs to determine if anthelmintic treatment was warranted, sources of livestock drinking water, awareness of internal protozoan and coccidian parasites (Cryptosporidium, Giardia and Eimeria) and knowledge as to whether these parasites were known to cause disease in sheep flocks within a district. A copy of the survey constructed for this study is available in the Appendix of this thesis (Chapter Thirteen).

“Evidence of recent diarrhoea” was defined in the questionnaire as active diarrhoea (loose or liquid faeces) or fresh faecal soiling of the breech fleece, where the breech fleece faecal soiling scores ranged from score three to score five using standard breech fleece faecal soiling scores (Australian Wool Innovation et al., 2007). A graphical representation
of standard breech fleece faecal soiling scores was included in the survey and is presented in Figure 3.3 in Chapter Three: Materials and Methods.

Scenarios were presented whereby 5%, 25% or 50% of the respondent’s meat lamb flock were affected with diarrhoea and the response options included: doing nothing, monitor flock to see if the incidence of diarrhoea becomes worse, conduct flock WEC, administer an anthelmintic treatment only to lambs with evidence of diarrhoea, administer an anthelmintic treatment to the entire flock or have the problem investigated by a veterinarian. More than one response could be selected for each scenario. These scenarios were included to assess meat lamb producers’ management and anthelmintic treatment responses to different, defined diarrhoea scenarios described above.

The questionnaire was trialled with 13 sheep farmers at the Muchea Livestock Saleyards (Muchea, Western Australia) in November 2010. Then 336 surveys were distributed by post (n=264) or electronic-mail (n=72) to sheep enterprises throughout south-west Western Australia via a Merino breeding alliance, two sheep meat processors, a livestock exporter and an agricultural lobbying group. A postage paid return-addressed envelope was included with the questionnaire. No telephone contacting or extra incentives (such as raffle prize draws) were offered to increase reply rate.

4.2.3 AGRICULTURE ZONES

Responses were categorised into six agricultural zones, depending on where respondents were located. These zones are shown in Figure 3.1 in Chapter Three: Materials and Methods. These agricultural zones were developed from statistical analyses of crop performances, average annual rainfall and length of the growing season. Region cells were coded by location ranging from 1 (north) to 5 (south) and by annual rainfall
categorised as being very high (>700mm), high (451–700mm), medium (325–450mm) and low (<325mm) (VH, H, M and L) (Figure 3.1) (Garlinge, 2005). Generally, moving north shortens the length of the growing season and moving east (inland) reduces the average annual rainfall. Quantum GIS mapping software (Quantum Geographic Information System, Version 1.6.0) was used to map the locations of survey farm respondents (Figure 4.1).

Figure 4.1: Quantum Geographical Information System Distribution of meat lamb farm enterprises surveyed (red star labels) in the southern Western Australia.

4.2.4 STATISTICAL ANALYSIS

The Statistical analysis was performed using SPSS Statistics 17.0 (Statistical Package for the Social Sciences) for Windows (SPSS inc. Chicago, USA). Reported diarrhoea prevalences (including 95% confidence intervals) were calculated using the exact binomial method for all respondents and for individual agriculture zones (Thrusfield, 2007). Pearson’s chi-squared test or Fisher’s exact two-sided test for independence were used to determine if management practices were associated with differences in the reported prevalence of diarrhoea.
Binary logistic regression (multivariable analysis) model was conducted to examine the association of reported diarrhoea (outcome variable) with covariate variables including; winter or autumn lambing, an anthelmintic administered to pregnant ewes, an anthelmintic administered to lambs, WEC utilised to determine if anthelmintic treatment is warranted (yes or no), presence of cattle on property and if the property was located in agricultural zone 5 or 6. In addition, the sources of livestock water (dam, river/creek, bore, or scheme water) were included as covariate factors. Backward elimination was used to determine which covariate factors were removed from the binary logistic regression model, until only significant factors remained. The likelihood-ratio test statistic was calculated to determine the significance at each regression step of the model, followed by building and testing the goodness-of-fit for the logistic regression models. The level of significance for a factor to remain in the final model was set at 5%, with variables that were included in the final model checked for collinearity as described by Stern (2010). Variables with tolerance values >0.1 were considered not to be correlated with other variables and therefore retained in the final model (Stern, 2010).

Univariable analyses (ANOVA) were conducted with least significant difference post-hoc tests to determine if the observed diarrhoea, proportion of lambs effected with diarrhoea or utilisation of WEC, were associated with farm average annual rainfall (mm/annum) or the agricultural zone location.

4.3 RESULTS

4.3.1 RESPONSE RATE

Of the 336 questionnaires distributed to farmers, 164 (49%) replies were received, of which 139 (41.1%) valid, with their distribution across southern Western Australia
illustrated in Figure 4.1. The response rate was 139/264 (53%) for mailed questionnaires, 11/59 (19%) for e-mailed questionnaires and 13/13 (100%) for personally distributed questionnaires.

4.3.2 MEAT LAMB ENTERPRISE CHARACTERISTICS

Farm characteristics, number of producer responses, annual rainfall and other farm information is outline in Table 4.1, with meat lamb respondents grouped by agricultural zones.

4.3.3 REPORTED DIARRHOEA PREVALENCE IN MEAT LAMBS

The reported diarrhoea prevalence in meat and lamb flocks during 2010 is detailed in Table 4.2. The reported diarrhoea prevalence in agricultural zone 6 (100%) was different to all other agriculture zones ($P<0.05$), except to zone 5.

4.3.4 PROPORTION OF MEAT LAMBS PER ENTERPRISE REPORTED WITH DIARRHOEA

The proportion of meat lambs per enterprise showing recent evidence of diarrhoea between 2–30%, with the highest mean proportion of such lambs with diarrhoea recorded in agricultural zone 6 (10.6%) and this was higher than all other zones ($P=0.043$) (Table 4.2).

Diarrhoea was most commonly first observed in the months of August (48.9%) or September (27.8%). The proportion of respondents reporting diarrhoea first observed in these months was different to June (3.3%), July (10.0%) and October (10.0%) ($P<0.05$).
Mean annual rainfall was higher on farms that reported observing diarrhoea in meat lambs (453 ± 12mm per annum) compared to those farms where no diarrhoea was reported (403 ± 10mm per annum; \( P=0.002 \)).
Table 4.1: Meat lamb farm information from survey respondents across different agricultural zones in Western Australia.

<table>
<thead>
<tr>
<th>Agricultural zone*</th>
<th>Number of farms</th>
<th>Annual rainfall per year (mm)</th>
<th>Area cropped (hectares)</th>
<th>Area grazed (hectares)</th>
<th>Ewes mated (n)</th>
<th>Number of ewes mated to terminal sires</th>
<th>Percentage of ewes mated to terminal sires (%)</th>
<th>Farms grazing cattle (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>North West</td>
<td>8</td>
<td>439 ± 29</td>
<td>3095 ± 1127</td>
<td>2088 ± 870</td>
<td>3163 ± 1082</td>
<td>1863 ± 638</td>
<td>64.2 ± 11.1</td>
<td>4</td>
</tr>
<tr>
<td>Central</td>
<td>49</td>
<td>393 ± 9</td>
<td>1656 ± 224</td>
<td>1335 ± 190</td>
<td>2236 ± 267</td>
<td>1402 ± 194</td>
<td>65.6 ± 3.7</td>
<td>7</td>
</tr>
<tr>
<td>South West</td>
<td>57</td>
<td>470 ± 9</td>
<td>678 ± 75</td>
<td>940 ± 58</td>
<td>2401 ± 232</td>
<td>1018 ± 98</td>
<td>49.9 ± 3.2</td>
<td>10</td>
</tr>
<tr>
<td>North East and Central</td>
<td>8</td>
<td>296 ± 7</td>
<td>3067 ± 991</td>
<td>597613 ± 35544</td>
<td>3350 ± 512</td>
<td>1850 ± 329</td>
<td>61.7 ± 12.2</td>
<td>3</td>
</tr>
<tr>
<td>Lakes/Mallee</td>
<td>8</td>
<td>428 ± 34</td>
<td>1631 ± 507</td>
<td>1925 ± 333</td>
<td>2863 ± 407</td>
<td>1394 ± 151</td>
<td>54.3 ± 8.5</td>
<td>1</td>
</tr>
<tr>
<td>South Coast</td>
<td>9</td>
<td>578 ± 26</td>
<td>972 ± 293</td>
<td>1158 ± 197</td>
<td>2656 ± 353</td>
<td>1389 ± 185</td>
<td>58.1 ± 9.2</td>
<td>3</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td></td>
<td>436 ± 8</td>
<td>1374 ± 138</td>
<td>4620 ± 2244</td>
<td>2485 ± 154</td>
<td>1296 ± 92</td>
<td>57.7 ± 2.3</td>
<td>0.20</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>139</td>
<td>-</td>
<td>190,919</td>
<td>642,164</td>
<td>345,340</td>
<td>180,112</td>
<td>-</td>
<td>28</td>
</tr>
</tbody>
</table>

* Agricultural zone boundaries are shown in Figure 4.2
Table 4.2: Survey replies from meat lamb farms by agricultural zones, with diarrhoea prevalence and proportion of their lamb flocks that experienced active or recent evidence of diarrhoea.

<table>
<thead>
<tr>
<th>Agricultural zone*</th>
<th>Number (n)</th>
<th>Respondents reporting diarrhoea in lambs</th>
<th>Average proportion of lamb flock with evidence of diarrhoea (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 North West</td>
<td>8</td>
<td>4</td>
<td>50.0&lt;sup&gt;A&lt;/sup&gt; (15.7, 84.3)</td>
</tr>
<tr>
<td>2 Central</td>
<td>49</td>
<td>30</td>
<td>61.2&lt;sup&gt;A&lt;/sup&gt; (46.2, 74.8)</td>
</tr>
<tr>
<td>3 South West</td>
<td>57</td>
<td>38</td>
<td>66.7&lt;sup&gt;A&lt;/sup&gt; (52.9, 78.6)</td>
</tr>
<tr>
<td>4 North East and Central</td>
<td>8</td>
<td>3</td>
<td>37.5&lt;sup&gt;A&lt;/sup&gt; (8.5, 75.5)</td>
</tr>
<tr>
<td>5 Lakes/Mallee</td>
<td>8</td>
<td>6</td>
<td>75.0&lt;sup&gt;AB&lt;/sup&gt; (34.9, 96.8)</td>
</tr>
<tr>
<td>6 South Coast</td>
<td>9</td>
<td>9</td>
<td>100.0&lt;sup&gt;B&lt;/sup&gt; (66.4, 100.0)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>139</strong></td>
<td><strong>90</strong></td>
<td><strong>Mean</strong> 64.8 (56.2, 72.7)</td>
</tr>
</tbody>
</table>

<sup>*Agricultural zones and their boundaries are shown in Figure 4.2.</sup>

<sup>AB</sup> Values in columns with different superscripts are significantly different (<i>P</i>&lt;0.05).

4.3.5 DIARRHOEA RISK FACTOR ANALYSES

Six management factors were significantly (<i>P</i>&lt;0.05) associated with the risk of diarrhoea using univariable analyses. These were; (1) property location in agricultural zone 5 or 6, (2) an anthelmintic treatment administered to lambs, (3) protozoa or coccidia known to cause disease in sheep farms within enterprise district, (4) livestock water sourced from a dam, (5) comprehensive water scheme or (6) bore (Table 4.3).

Multivariable analyses by binary logistic regression identified four factors that were significantly (<i>P</i>&lt;0.05) associated with the risk of diarrhoea. Livestock water sourced from a dam and property location in either agricultural zone 5 or 6, both increased the risk of
reporting diarrhoea (Table 4.4). Livestock water sourced from either scheme or bore both decreased the risk of diarrhoea (Table 4.4).

**Table 4.3:** Univariable associations between management practices with the risk of diarrhoea in meat lamb farms from Western Australia.

<table>
<thead>
<tr>
<th>Management variable</th>
<th>Percentage of respondents that observed diarrhoea</th>
<th>Odds ratio (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farm property located in agricultural zone 5 or 6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>88.2</td>
<td>4.70 (1.03, 21.49)</td>
<td>0.030*</td>
</tr>
<tr>
<td>No</td>
<td>61.5</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Lambing season</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autumn</td>
<td>62.3</td>
<td>1.19 (0.58, 2.43)</td>
<td>0.630</td>
</tr>
<tr>
<td>Winter</td>
<td>66.3</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Anthelmintic treatment administered to pregnant ewes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>66.2</td>
<td>1.14 (0.57, 2.28)</td>
<td>0.715</td>
</tr>
<tr>
<td>No</td>
<td>63.2</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Anthelmintic treatment administered to lambs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>70.9</td>
<td>2.72 (1.25, 5.93)</td>
<td>0.011</td>
</tr>
<tr>
<td>No</td>
<td>45.2</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Faecal worm egg count used to determine if anthelmintic treatment is warranted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>67.4</td>
<td>1.40 (0.68, 2.90)</td>
<td>0.361</td>
</tr>
<tr>
<td>No</td>
<td>59.6</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Cattle grazed on farm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>57.1</td>
<td>0.67 (0.29, 1.55)</td>
<td>0.346</td>
</tr>
</tbody>
</table>
Protozoa or coccidia known to cause disease in sheep or lambs in nearby district.

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>85.7</td>
<td>4.09 (1.33, 12.59)</td>
<td>0.009</td>
</tr>
<tr>
<td>No/Unsure</td>
<td>59.5</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

Livestock water from a dam

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>88.9</td>
<td>152.0 (32.1, 719.0)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>No</td>
<td>5.0</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

Livestock water from a river or creek

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>83.3</td>
<td>2.82 (0.32, 24.88)</td>
<td>0.330*</td>
</tr>
<tr>
<td>No</td>
<td>63.9</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

Livestock water from a bore

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>43.8</td>
<td>0.32 (0.14, 0.72)</td>
<td>0.005</td>
</tr>
<tr>
<td>No</td>
<td>71.0</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

Livestock water from a scheme

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>42.3</td>
<td>0.14 (0.06, 0.34)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>No</td>
<td>75.5</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

* Fisher’s exact test (two-sided significance).
Table 4.4: Binary logistic regression model of management factors associated with the risk of active diarrhoea in meat lamb flocks in Western Australia.

<table>
<thead>
<tr>
<th>Covariate variables</th>
<th>( \beta ) - estimates</th>
<th>Odds Ratio (95% CI)</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farm property from agricultural zone 5 or 6</td>
<td>2.07</td>
<td>7.92 (1.82, 45.27)</td>
<td>0.020</td>
</tr>
<tr>
<td>Livestock water from a dam</td>
<td>4.73</td>
<td>117.1 (18.19, 754.79)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Livestock water from a bore</td>
<td>-1.98</td>
<td>0.45 (0.16, 0.89)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Livestock water from a scheme</td>
<td>-3.04</td>
<td>0.28 (0.08, 0.46)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Constant</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Hosmer and Lemeshow statistic = 0.831, Cox and Snell \( R^2 \) value = 0.295 and Nagelkerke \( R^2 \) value = 0.406.

All variables had tolerance values >0.1

4.3.6 SHEEP MANAGEMENT PRACTICES RELEVANT TO DIARRHOEA

A total of 71/139 (51.1%) respondents administered an anthelmintic treatment to pregnant ewes before lambing and 103/139 (74.1%) administered an anthelmintic treatment to meat lambs. Respondents that administered an anthelmintic treatment to lambs, reported a higher proportion of lambs with diarrhoea (7.5 ± 0.66%) compared to respondents that didn’t administer an anthelmintic treatment (4.4 ± 1.36%; \( P=0.041 \)).

To determine if an anthelmintic treatment was warranted, 34.5% of respondents reported never using WECs, 39.6% occasionally used WECs, 21.6% usually used WECs and 4.3% of respondents always used WECs, to determine if an anthelmintic treatment was warranted. Increased use of WECs was associated with an increased farm average annual rainfall, whereby average annual rainfall for those respondents, reporting to never...
(402 ± 12.2mm), occasionally (432 ± 11.4mm), usually (477 ± 15.4mm) and always (536 ± 24.9mm) utilised WECs, were all different to one another ($P<0.001$).

4.3.7 RESPONDENT AWARENESS OFPROTOZOAN AND COCCIDIAN PARASITES

Overall, 47.5% respondents were not aware and 32.4% respondents were unsure about protozoan or coccidian parasites ($Cryptosporidium$, $Giardia$ and $Eimeria$) being a contributing cause of sheep disease within their district. Specifically, 18/139 (12.9%) were aware of $Eimeria$, 14/139 (10.1%) were aware of $Cryptosporidium$ and 20/139 (14.4%) were aware of $Giardia$, with 11/139 (8.0%) being aware of two or more of these above parasites and 6/139 (4.3%) aware of all three parasites.

4.3.8 RESPONDENT RESPONSES TO DEFINED DIARRHOEA SCENARIOS

Across the three diarrhoea scenarios presented (5%, 25% and 50% of respondent meat lamb flock affected by diarrhoea), 21/139 (15.1%) respondents elected to administer an anthelmintic treatment to the entire lamb flock for all of the three scenarios; 63/139 (45.3%) elected to administer an anthelmintic treatment to the entire lamb flock for only one scenario; and 15/139 (10.8%) never elected to administer an anthelmintic treatment for any scenario (Table 4.5). Overall, 64/139 (46.0%) and 90/139 (64.7%), elected not to conduct a flock WEC and not to consult a veterinarian respectively, regardless of the scenario presented (Table 4.5). A total of 23/139 (16.6%) respondents made additional comments indicating that they would either provide oaten hay supplementation or move the flock to a different paddock, if 25% or 50% of their lamb flock was effected with diarrhoea.
Table 4.5: Farmer responses towards different diarrhoea scenarios, whereby 5%, 25% and 50% of a meat lamb flock had active diarrhoea or evidence of recent diarrhoea (n=139 responses).

<table>
<thead>
<tr>
<th>Percentage of lamb flock with diarrhoea (%)</th>
<th>Monitor to see if diarrhoea incidence become worse</th>
<th>Conduct flock WEC</th>
<th>Administer anthelmintic treatment</th>
<th>Veterinary investigation</th>
<th>Do nothing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(n)</td>
<td>Entire flock</td>
<td>Diarrhoeic or fleece soiled lambs only</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>94</td>
<td>13</td>
<td>22</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>25</td>
<td>31</td>
<td>60</td>
<td>69</td>
<td>26</td>
<td>2</td>
</tr>
<tr>
<td>50</td>
<td>1</td>
<td>30</td>
<td>114</td>
<td>6</td>
<td>49</td>
</tr>
</tbody>
</table>

4.4 DISCUSSION

This is the first epidemiological investigation of diarrhoea reported for meat lamb flocks on-farm in southern Australia. A novel finding in this study was the association between livestock water sources and the reported prevalence of diarrhoea, whereby lambs which drank water sourced from a dam were more than a 100 times more likely to experience diarrhoea compared to other water sources (Table 4.3 and Table 4.4). It is possible that the lambs drinking from open water sources (dams, rivers or creeks) had increased risk of exposure to faecal pathogens (including protozoa, bacteria and viruses), compared to lambs drinking water supplied from either bore or scheme sources. Faecal material, fertilisers and pesticide residues can be washed from pastures into open water sources following moderate to high rainfall events and this has potentially deleterious effects on livestock water quality (Coddington, 1992; Sharpley and Withers, 1994; Hooda et al., 2000; Smith and Frost, 2000; Bodley-Tickell et al., 2002; Delin and Landon, 2002; Chadwick et al., 2008; Edwards et al., 2008).
In contrast, bore and scheme water are protected to a greater extent from contaminants by storage in underground aquifers or via managed catchments, tanks and troughs. With respect to ground water (bore or scheme), surface water movement through the soil has been reported to filter some impurities (fertiliser and pesticide residues) and pathogens as the water passes through different soil layers and pores in the infiltration process. The distance and speed that water travels through a soil profile depends upon soil structure, soil particle size, soil pore size and the depth of the aquifer supplying either the bore or scheme water source and all these factors influence the filtration of pathogens and residues (Stevik et al., 1999; Unc and Goss, 2004; Mosaddeghi et al., 2009; Schinner et al., 2010).

Diarrhoea was most commonly reported to have been first observed in meat lambs in late winter (August) and early spring (September). Rainfall events reported across the south-west land division in early July, mid and late August 2010 (Australian Bureau of Meterology, 2011b), were likely to have contributed to increased pasture growth, increased distribution of strongylid nematode larvae over pastures and increased surface water run-off from pastures into dams, rivers and creeks. Climatic conditions during August and September are also favourable for the survival of infectious parasite stages, including strongylid nematode third stage larvae (Dobson et al., 1990d; Marley et al., 2006; Moss and Bray, 2006) and Cryptosporidium, Giardia and Eimeria (oo)cysts (Robertson et al., 1992; Fayer et al., 1996). The actual (observed) annual rainfall across the survey region was below average during the 2010 survey period (Figure 4.2), with winter rainfall being the second driest on record and spring rainfall the fifth driest on record for the region (Australian Bureau of Meterology, 2011b). Agriculture zones 5 and 6 were not as severely impacted by the reduced annual rainfall when compared to the other agriculture zones.
(particularly zones 2 and 3) in 2010 (Figure 4.2), and this is potentially why farming properties located in zones 5 and 6 had an increased risk of reporting diarrhoea in meat lambs.

Figure 4.2: Annual rainfall decile ranges across southern Western Australia from January 1\textsuperscript{st} to December 31\textsuperscript{st} 2010 (Australian Bureau of Meterology, 2011a).

The majority of respondents had administered an anthelmintic treatment to lambs and respondents who observed diarrhoea were 2.7 times more likely to have administered an anthelmintic treatment to lambs, than respondents that did not. It was likely that for those respondents located in districts where internal parasites are a well recognised disease risk in sheep, that they would be more likely to implement strategic (preventive) or
tactical (in response to suspected helminthosis) anthelmintic treatments. It is also possible that that these same respondents elected to administer an anthelmintic to lambs in response to an outbreak of diarrhoea and/or fresh breech fleece faecal soiling (Besier and Love, 2003; Coles et al., 2006; Besier, 2008; Woodgate and Besier, 2010). This suggestion was supported by the finding that 16-82% of respondents reported that they would elect to treat the whole lamb flock, where 5%, 25% or 50% of the flock were observed with diarrhoea. However, an investigation of slaughter lambs at abattoirs showed that observation of active diarrhoea or fresh faecal soiling was a poor predictor, as to which consigned groups had high flock WECs (Jacobson et al., 2009a).

Over a third of respondents reported never using WECs to determine if an anthelmintic treatment was warranted and nearly half of respondents reported that they wouldn’t elect to conduct a flock WEC where 5%, 25% or 50% of the flock was effected with diarrhoea (Table 4.5). Farmers that occasionally, usually or always used WECs for planning parasite control (determining if an anthelmintic treatment was warranted), were from districts with higher average annual rainfall and therefore likely to have a higher risk of helminthosis, compared to those districts with lower average annual rainfall. Anthelmintic resistance poses an ongoing challenge to sheep enterprises (Besier and Love, 2003; Gillear, 2006; Beraldi et al., 2008; Greer et al., 2009; Jackson et al., 2009; Mitchell et al., 2010; Sargison et al., 2010; Sutherland et al., 2010) and an increased utilisation of WEC testing by farmers, as a means of determining if anthelmintic treatments are justified, may aid in reducing treatment frequency, increasing refugia and delaying resistance development to treatments (Dobson et al., 2001; Besier and Love, 2003; Besier, 2008; Woodgate and Besier, 2010).
Less than 2% of respondents reported that they would elect to consult a veterinarian if 5% or 25% of their lambs had active or recent evidence of diarrhoea and only 35% of respondents reported that they would elect to consult a veterinarian if 50% of their lambs had active or recent signs of diarrhoea (Table 4.5). Respondents were 4.1 times more likely to report diarrhoea in their lamb flocks, when they were aware that protozoa and/or coccidia were known causes of disease in sheep within their district. This suggests that respondents reporting diarrhoea in their flocks are potentially more aware of the infectious agents that are associated with diarrhoea. Strengthening communication between farmers and veterinarians may be one way to improve the uptake of sustainable parasite control programmes, which incorporate utilising flock WECs and improving the probability of detecting other infectious agents associated with diarrhoea and reduced sheep productivity.

A survey questionnaire was considered the most practical method to obtain the information, with the questionnaires designed to communicate clearly to respondents what the researchers are asking for and allow accurate retrieval of data. Although a graphical representation of recent evidence of diarrhoea was included in the questionnaire, data in this research was quite subjective depending upon the different experiences of respondents with respect to the detection of active or recent evidence of diarrhoea and the proportion of lambs they observed with diarrhoea.

This study accounted for ~11% of the 1,316 farms reported to have a meat lamb enterprise. The questionnaire was designed with the aim of maximising response rate, by making it concise and limiting the complexity of questions. As a result, limitations of this study included not clarifying the type of anthelmintic treatment administered to ewes and
lambs and not determining the causes of diarrhoea, as a large number of infectious and non-infectious agents have been associated with diarrhoea in lambs: strongylid nematodes, Cryptosporidium, Giardia and Eimeria (Gregory and Catchpole, 1990; Olson et al., 1995; Sargison, 2004; Aloisio et al., 2006), bacteria (Campylobacter spp., Yersinia spp. and Salmonella spp.), as well as viruses (Skirrow, 1994; Belloy et al., 2009) and fungal endophytes (Eerens et al., 1998).

4.5 CONCLUSION

Diarrhoea was reported in 65% of the surveyed meat lamb enterprises from southern Western Australia in 2010, with the source of livestock drinking water identified as an important diarrhoea risk factor. An increased anthelmintic treatment frequency was observed in those flocks with diarrhoea and this practice increases the risk of anthelmintic resistance development. Improving the availability of cost-effective diagnostic tools may strengthen our understanding of the risk factors associated with diarrhoea in lambs and reveal if there are more options to limit both flock welfare and productivity consequences associated with diarrhoea.
CHAPTER 5: DIFFERENT STRONGYLID INFECTION DETECTION METHODS

COMPARISON OF MOLECULAR AND MCMASTER MICROSCOPY TECHNIQUES TO CONFIRM THE PRESENCE OF NATURALLY ACQUIRED STRONGYLID NEMATODE INFECTIONS IN SHEEP


5.1 INTRODUCTION

Strongylid nematodes are an important cause of income loss in sheep enterprises as a consequence of reduced flock productivity and increased costs associated with anthelmintic treatments. In southern Australia, the most economically important sheep strongylid genera are *T. circumcincta*, *Trichostrongylus* spp. and *H. contortus*, with *C. ovina*, *Oesophagostomum* spp. and *Nematodirus* spp. also commonly present as part of mixed infections (Besier and Love, 2003). Accurate diagnosis and quantification of strongylid infection is pivotal for both effective control programs and monitoring anthelmintic treatment efficacy (Besier and Love, 2003; Woodgate and Besier, 2010). Species-specific diagnosis has important implications for anthelmintic treatment decisions, surveillance of anthelmintic resistance and monitoring strongylid epidemiology demographics in different geographical locations.

The McMaster WEC flotation technique (Whitlock, 1948) is widely utilised for the diagnosis and quantification of strongylid worm infections in sheep. The WEC technique is
a useful indicator for predicting patent worm burdens, particularly in younger sheep (Kingsbury, 1965), but cannot distinguish between strongylid species without the use of larval culture. Larval cultures are time consuming, require skilled laboratory staff, depend on strictly controlled culture conditions (temperature and humidity) to prevent a species biased culture and require relatively large volumes of faecal material. As a result, larval cultures are typically only performed on pooled faecal samples (Dobson et al., 1992).

Polymerase chain reaction assays have been developed to detect patent strongylid species infections in sheep by targeting genetic markers within the internal transcribed spacer (ITS-2) region of nuclear ribosomal DNA (Bott et al., 2009). These assays have been used to detect strongylid DNA extracted from worm eggs, following egg purification from sheep faeces by sodium nitrate flotation and column-purification (Bott et al., 2009; Roeber et al., 2011). Comparisons of the level of agreement between the McMaster WEC and PCR diagnostic tests, which screen genomic DNA extracted directly from faeces, have not been reported.

### 5.1.1 AIMS AND HYPOTHESES

Therefore, the aim of this study was to:

1. Utilise a commercial DNA isolation kit to extract genomic DNA directly from raw, unprocessed sheep faeces.
2. Compare the level of agreement between PCR assays (screening faecal DNA extracts) and McMaster WEC (microscopy) results for identifying patent strongylid infections in lambs.
3. Examine the strongylid epidemiology in each flock
The hypotheses of this experiment were that:

1. PCR assays successfully amplify L₃ spiked positive faecal samples.
2. PCR (screening DNA extracted directed from faeces) and McMaster WEC (microscopic) results for identifying patent strongylid infections have a high level of agreement.

5.2 MATERIALS AND METHODS

5.2.1 GEOGRAPHICAL STUDY SITES, FAECAL SAMPLE COLLECTION AND ANTHELMINTIC TREATMENTS

This experiment was approved by the Murdoch University Animal Ethics Committee (permit R2369/10). Samples were taken from four sheep farms located in southern Western Australia (Boyup Brook, Kojonup, Pingelly and Arthur River), in a geographical region with a Mediterranean environment characterised by hot, dry summers and cool, wet winters (Hill et al., 2004; Moeller et al., 2008). Average annual rainfall for the four sites ranged between 420 – 520mm per annum. The lambs used in the study were grazed on a single paddock with annual pastures, where annual rye-grasses (Lolium spp.) and subterraneum clover (Trifolium subterraneum) were the main species.

A total of 858 faecal samples were collected over two separate sampling occasions when lambs were approximately 2 – 3 months old (first sampling) and 4 – 5 months old (second sampling). Lambs at Pingelly and Arthur River were treated with 12mg abamectin (Virbamec Oral Plus Selenium, Virbac Australia), while those lambs at Boyup Brook and Kojonup were treated with 12.5mg of moxidectin (Cydectin Weanerguard with Selenium and Vitamin B12, Virbac Australia) immediately after the first sampling. The number of
days between the first and second samplings was 35, 29, 40 and 31 for Boyup Brook, Kojonup, Pingelly and Arthur River farms respectively.

Negative control faecal samples were collected rectally from 6-year-old Merino ewes seven days post-treatment with 2.5mg/kg Monepantel (Zolvix, Novartis Australia) administered according to the heaviest ewe live weight.

5.2.2 FAECAL WORM EGG COUNTS AND LARVAL CULTURES

Faecal worm egg counts were performed within two days of collection using a modified McMaster WEC flotation technique with a detection level/multiplication factor of 50epg (Lyndal-Murphy, 1993). Larval cultures were performed according to the Australian Standard Diagnostic Techniques for Animal Diseases Manual (Lyndal-Murphy, 1993) on pooled faecal samples from only the Boyup Brook and Kojonup flocks.

5.2.3 DNA EXTRACTION

The PCR-positive controls were created using strongylid third-stage larvae (L₃) suspensions. Larvae were collected from larval cultures of fresh sheep faeces and 200µl aliquot L₃ suspensions were collected for each of the following strongylid species; *T. circumcincta*, *Trichostrongylus colubriformis*, *H. contortus*, *C. ovina* and *Oesophagostomum venulosum*. Each larvae suspension from the five strongylid genera had DNA extracted from larval tissues (Figure 5.1 [A]). Suspensions of 100µL were created to contain equal proportions of strongylid genera L₃ and used to spike the positive controls (Figure 5.1[B]). Unspiked, negative controls (ewe faecal samples providing three consecutive WEC<50epg; n=96) and spiked positive controls (ewe faecal samples providing three consecutive WEC<50epg, spiked with a 100µL suspension containing equal proportions of strongylid species L₃; n=96) were generated to ascertain whether PCR
inhibition was observed for any of the strongylid species-specific PCR assays (Figure 5.1 [B]).

Genomic DNA was extracted from lamb faecal samples within seven days of collection by using Power Soil DNA Isolation Kits (MolBio, West Carlsbad, California, USA) (Figure 5.1 [C]). A sub-sample comprising of 250–300mg was taken from the centre of each faecal sample. Previously reported minor modifications to the manufacturer’s protocol were made and are detailed in the thesis Appendix (Chapter Thirteen).

5.2.4 PCR AMPLIFICATION

Conventional PCR assays were performed for each of the following strongylid species; *T. circumcincta*, *Trichostrongylus* spp., *H. contortus* and *C. ovina*, as described by a previous study (2009). Individual species-specific forward primers (TEL, TRI, HAE and CHO) designed for the second internal transcribed spacer (ITS-2) of ribosomal DNA and the reverse primer (NC2) located at the 5’-region of the 28S rRNA gene, were used to detect each of the above species. The primer pair TRI-NC2, was capable of detecting all four major *Trichostrongylus* species (*T. colubriformis*, *T. axei*, *T. vitrinus* and *T. rugatus*) and in addition *Oesophagostomum columbianum* and *Oesophagostomum venulosum* (Bott et al., 2009). The thermocycling conditions used for each PCR assay are presented in an earlier study (2009). The PCR reactions were performed using 1µL of DNA in a 25µL reaction containing 1 x PCR buffer, 2.5mM MgCl₂, 0.4mM dNTPs, 0.80µM of each primer and 0.04U/µL of *kapa* Taq DNA Polymerase (Kapa Biosystems, Cape Town, South Africa). Negative (no DNA template added) and positive (genomic DNA from L₃ tissue extractions) controls were included for all strongylid species PCR assays.
For any samples that tested McMaster WEC-positive and PCR-negative, a 10µL genomic DNA extract of the sample was spiked with 1µL aliquots of DNA from each of the five strongylid species (isolated from the L₃ DNA tissue extractions). From these 15µL spiked mixtures, a 1µL aliquot was then re-screened with each strongylid species-specific PCR assay to detect any inhibition.

5.2.5 SEQUENCE ANALYSIS

The PCR 1% agarose gel product for samples that tested PCR-positive and McMaster WEC-negative (n=17 at the first sampling and n=7 at the second sampling) were purified using an UltraClean™ DNA Purification Kit (MolBio, West Carlsbad, California, USA) and sequenced using an ABI Prism™ Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) on an Applied Biosystem 3730 DNA Analyzer. Sequence searches were conducted using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and nucleotide sequences were analysed using Chromas lite version 2.0 (http://www.technelysium.com.au). Subsequently these searches were aligned with reference genotypes to confirm positive identification for either *H. contortus*, *T. circumcincta*, *Trichostrongylus axei*, *Trichostrongylus colubriformis*, *C ovina*, *Oesophagostomum venulosum* or *Oesophagostomum columbianum* (GenBank accession numbers AJ57746.1, AJ577463.1, AY439026.1, EF427624, AY439021.1, Y10790.1 and AJ006150, respectively) using Clustal W (http://www.clustalw.genome.jp).
Figure 5.1: Methodology utilised to screen PCR positive controls [A], spiked and unspiked ewe control faecal samples [B] and on-farm lamb test samples [C].
5.2.6 STATISTICAL ANALYSIS

Statistical analyses were performed using SPSS Statistics 17.0 (Statistical Package for the Social Sciences) for Windows (SPSS inc. Chicago, USA). The sensitivity and specificity between PCR and McMaster WEC results were calculated for faecal samples collected rectally from lambs on-farm, with their infection status unknown. Kappa statistic was calculated at each sampling to assess the level of agreement between the McMaster WEC and PCR test results. Either Pearson's chi-squared test or Fisher's exact two-sided test for independence was used to determine if significant differences existed between the proportions of mixed infections detected at each sampling within each flock. The mean number of strongylid species detected from each lamb was calculated using arithmetic means. Differences in the mean number of strongylid species detected per lamb were performed by univariable analyses (ANOVA) and least significant difference post-hoc tests.

5.3 RESULTS

5.3.1 PCR ASSAYS DIAGNOSTIC SENSITIVITY – SPIKED SAMPLES

The PCR assays had a diagnostic sensitivity of 100% for the 96 spiked positive controls and a specificity of 100% (95% CI: 96.2 – 100%) for the negative controls (n=96) providing three consecutive WEC<50epg (Table 5.1).
Table 5.1: Comparison of spiked positives (n=96) and negative samples (n=96) by PCR strongylid nematode diagnostic assays.

<table>
<thead>
<tr>
<th></th>
<th>Spiked L₃ positives</th>
<th>Unspiked negatives</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR Positive</td>
<td>96</td>
<td>0</td>
<td>96</td>
</tr>
<tr>
<td>PCR Negative</td>
<td>0</td>
<td>96</td>
<td>96</td>
</tr>
</tbody>
</table>

Note: Spiked L₃ positives were lamb faecal samples that provided three consecutive WEC<50epg. These samples were spiked with 100µl suspensions of strongylid L₃ of all five nematode species screened for by all five species-specific PCR assays. Negative controls (unspiked) faecal samples also had genomic DNA extracted and were screened by each species-specific PCR assay.

5.3.2 AGREEMENT BETWEEN PCR ASSAYS AND WEC RESULTS

There was a high level of agreement between PCR and McMaster WEC with Kappa values of 0.93 (90.4 – 95.4%) at the first sampling and 0.97 (94.6 – 98.2%) at the second sampling (Table 5.2). For identifying lambs with WEC≥50 epg, the PCR assays had a sensitivity of 99.7% (98.2 – 100%, n=301) and 100% (98.3 – 100%, n=221) and a specificity of 91.4% (85.1 – 95.6%, n=128) and 96.6% (93.2 – 98.6, n=208) at the first and second samplings, respectively (Table 5.2).

One sample (0.1%) was McMaster WEC-positive (50 epg) and PCR-negative. Separate spiked DNA extracts from this same sample were screened by PCR for the respective strongylid genera DNA to test for inhibition. This spiked sample amplified in accordance with positive controls for all species-specific PCRs.

Eighteen (2.1%) samples that were McMaster WEC-negative and PCR-positive were re-screened by PCR for all five strongylid species and sequenced. The sequenced products T. circumcincta (n=3), Trichostrongylus colubriformis (n=2), C. ovina (n=4) and
Oesophagostomum venulosum (n=8) were aligned with reference sequences on GenBank and were 100% identical.

**Table 5.2:** Comparison of the McMaster WEC (microscopy) and PCR diagnostic assays for the identification of strongylid positive or negative faecal samples.

<table>
<thead>
<tr>
<th>First sampling</th>
<th>Second sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>McMaster WEC</td>
</tr>
<tr>
<td>PCR assays</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>300</td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>301</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PCR assays</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>201</td>
<td>7</td>
<td>208</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>221</td>
<td>221</td>
</tr>
<tr>
<td>Total</td>
<td>201</td>
<td>228</td>
<td>429</td>
</tr>
</tbody>
</table>

### 5.3.3 STRONGYLID NEMATODE PREVALENCE AND SPECIES DETECTED BY PCR AND LARVAL CULTURE

Overall a total of 502/858 (58.5%) samples were McMaster WEC-positive (WEC≥50 epg) and 519/858 (60.5%) samples PCR-positive for at least one strongylid species (Table 5.3). There was no significant difference in the number of strongylid-positive samples identified by each of the two diagnostic tests. Overall prevalence of patent strongylid infections across all four flocks was 358/429 (83%) detected by PCR (lambs positive for at least one strongylid species on at least one sampling) and 351/429 (81%) detected by McMaster WEC (lamb WEC≥50 epg on at least one sampling).

Across all four flocks, the strongylid species most commonly identified by PCR were *T. circumcincta* (40.2%), *Trichostrongylus* spp. (24.7%), *C. ovina* (15.6%) and *Oesophagostomum* spp. (10.1%), with *H. contortus* only identified from Boyup Brook lambs (Table 5.3, Figure 5.2). Larval culture results for Boyup Brook and Kojonup flocks are
shown in Figure 5.3. The total numbers of strongylid species identified by PCR per lamb are shown in Figure 5.4. Mixed infections were identified in 35.3% and 17.0% of lambs at the first and second sampling, respectively. The most common mixed infection for all flocks was that of *Trichostrongylus* spp. and *T. circumcincta*. 
Table 5.3: Comparison of McMaster WEC (microscopy) and PCR diagnostic assays for the detection of patent strongylid infections in four lambs flocks.

<table>
<thead>
<tr>
<th>Site location</th>
<th>Total samples tested (n)</th>
<th>Mean ± SEM (epg)</th>
<th>Range (epg)</th>
<th>positive (n)*</th>
<th>Total H. contortus</th>
<th>T. circumcincta</th>
<th>Trichostrongylus spp.</th>
<th>C. ovina</th>
<th>Oesophagostomum spp.</th>
<th>Mixed infections a</th>
<th>Mean number of species detected per lamb ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boyup Brook</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First sampling</td>
<td>128</td>
<td>100 ± 13</td>
<td>0 – 750</td>
<td>81</td>
<td>84</td>
<td>9</td>
<td>63</td>
<td>44</td>
<td>14</td>
<td>13 (34.4%) a</td>
<td>1.11 ± 0.09 a</td>
</tr>
<tr>
<td>Second sampling</td>
<td>128</td>
<td>77 ± 12</td>
<td>0 – 700</td>
<td>59</td>
<td>62</td>
<td>8</td>
<td>50</td>
<td>34</td>
<td>10</td>
<td>4 (28.1%) a</td>
<td>0.83 ± 0.09 a</td>
</tr>
<tr>
<td>Kojonup</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First sampling</td>
<td>72</td>
<td>28 ± 5</td>
<td>0 – 150</td>
<td>26</td>
<td>27</td>
<td>0</td>
<td>23</td>
<td>8</td>
<td>8</td>
<td>4 (15.3%) a</td>
<td>0.60 ± 0.09 a</td>
</tr>
<tr>
<td>Second sampling</td>
<td>72</td>
<td>21 ± 4</td>
<td>0 – 150</td>
<td>24</td>
<td>24</td>
<td>0</td>
<td>18</td>
<td>11</td>
<td>3</td>
<td>3 (13.9%) a</td>
<td>0.50 ± 0.09 a</td>
</tr>
<tr>
<td>Pingelly</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First sampling</td>
<td>108</td>
<td>446 ± 45</td>
<td>0 – 3950</td>
<td>102</td>
<td>102</td>
<td>0</td>
<td>73</td>
<td>57</td>
<td>40</td>
<td>22 (69.4%) a</td>
<td>1.78 ± 0.08 a</td>
</tr>
<tr>
<td>Second sampling</td>
<td>108</td>
<td>73 ± 13</td>
<td>0 – 1100</td>
<td>56</td>
<td>57</td>
<td>0</td>
<td>28</td>
<td>24</td>
<td>15</td>
<td>8 (13.9%) a</td>
<td>0.70 ± 0.08 a</td>
</tr>
<tr>
<td>Arthur River</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First sampling</td>
<td>121</td>
<td>87 ± 8</td>
<td>0 – 450</td>
<td>92</td>
<td>98</td>
<td>0</td>
<td>42</td>
<td>18</td>
<td>36</td>
<td>27 (17.4%) a</td>
<td>1.05 ± 0.06 a</td>
</tr>
<tr>
<td>Second sampling</td>
<td>121</td>
<td>48 ± 7</td>
<td>0 – 450</td>
<td>62</td>
<td>65</td>
<td>0</td>
<td>48</td>
<td>16</td>
<td>8</td>
<td>6 (9.9%) a</td>
<td>0.65 ± 0.09 a</td>
</tr>
<tr>
<td>Total (%)</td>
<td>858</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>502</td>
<td>519 (60.5%)</td>
<td>17 (2.0%)</td>
<td>345 (40.2%)</td>
<td>212 (24.7%)</td>
<td>134 (15.6%)</td>
<td>87 (10.1%)</td>
</tr>
</tbody>
</table>

aValues in rows with different superscripts are significantly different within each farm (P<0.05).

*McMaster WEC-positive samples had a WEC≥50 epg.

a Mixed infections: lambs positive for two or more of strongylid species detected by the PCR diagnostic assays.
Figure 5.2: Number of lambs PCR-positive for each of the five strongylid species.

Figure 5.3: Strongylid nematode genera identified by larval culture differentiation from pooled faecal samples.
Figure 5.4: Number of strongylid species identified per lamb by PCR.

5.4 DISCUSSION

This study utilised molecular PCR assays that have been previously described (Bott et al., 2009) for detecting patent strongylid nematode infections from genomic DNA that was extracted directly from unprocessed sheep faeces. Other studies have demonstrated that these PCR assays can be used to identify naturally acquired strongylid infections following separation of strongylid worm eggs from faeces, by using sodium nitrate flotation and column-purification (Roeber et al., 2011). However in the present study, direct extraction of DNA from faeces by the use of a commercial DNA extraction kit successfully identified strongylid species-specific infection. The PCR diagnostic assays successfully identified all strongylid genera in faecal samples that were spiked with a L₃ suspension containing all five species. No PCR inhibition was detected in any of the L₃ spiked faecal
samples for all strongylid species-specific PCRs. Negative controls never tested positive for any of the strongylid species.

There was a high level of agreement between McMaster WEC and PCR diagnostic test results, suggesting that PCRs detecting DNA extracted directly from lamb faeces had a similar capacity to the traditional McMaster WEC technique for detecting patent strongylid infections. Faecal samples were collected from lambs on commercial sheep farms and post-mortem total worm count examinations were not performed, so the infection status of individual lambs was unknown. Studies that include total worm counts are necessary to confirm the PCR capacity for detecting patent strongylid infections and also to determine sensitivity and specificity for identifying infected sheep. If PCR is shown to have a higher level of sensitivity compared to traditional methods, then there may be an emerging recommendation to incorporate PCR assays for anthelmintic efficacy testing protocols where a high degree of precision for identifying infected sheep and strongylid species identification is required.

One (0.1%) sample was McMaster WEC-positive and PCR-negative. This may reflect the presence of strongylid species not screened for by PCR, but which have eggs indistinguishable from those of other strongylid species such as *Cooperia* spp., which has been reported in worm populations in southern Australian sheep flocks (Anderson, 1972). Another possible reason for this discrepancy is the uneven distribution of strongylid worm eggs within the faecal mass sample (Sinniah, 1982).

Eighteen (2.1%) samples were identified as PCR-positive but McMaster WEC-negative. False PCR-positive identification of patent strongylid infections appears unlikely, with a recent study finding that 100 sheep with a very low risk of nematode infection
(housed indoors and treated with anthelmintics) never tested positive in any species-specific PCR assays (Roeber et al., 2011). Lower detection limits for McMaster WEC (25 epg, 20 epg or 10 epg) could be compared to those PCR assays, to establish whether lower WEC detection limits decrease the number of samples identified as PCR-positive and McMaster WEC-negative.

The PCRs are capable of detecting DNA from strongylid species in sheep faeces, predominantly from worm eggs laid by established, mature females (Bott et al., 2009). However, DNA was extracted directly from faeces in this study and the PCR diagnostic assays were therefore presumably incapable of differentiating strongylid DNA originating from patent and non-patent infections (immature larvae and dead worm tissue present in faeces). Some lambs tested negative for strongylid infections while grazing pastures infested with larvae and this suggests that strongylid worm eggs are the likely main source of DNA in faecal DNA extractions. Neither McMaster WEC, nor PCR are capable of distinguishing between viable and non-viable strongylid eggs.

Although PCRs offer some advantages over traditional methods for identifying strongylid infected sheep, the assays are not quantitative and WECs are still required to quantify the magnitude of infections. The fact that PCRs are incapable of differentiating between patent and non-patent origins of DNA extracted directly from faeces, presents a possible disadvantage in detecting patent strongylid infections. However this attribute may be advantageous for the early detection pathogenic strongylid larvae species infesting those pastures with grazing sheep. Another disadvantage of the PCRs is that each strongylid species-specific PCR assay (except *Oesophagostomum* spp.) must be conducted separately and as a result a greater cost would be incurred associated with use
of more PCR reagents. Further modifications of the individual species-specific PCRs into a single, multiplex, quantitative qPCR assay would help facilitate the use of PCR for the routine diagnosis of helminthosis in sheep.

Direct DNA extraction allows for other internal pathogens (parasites, bacteria or viruses) to be screened for by utilising a similar molecular approach as conducted in this study. Moreover, the main advantage of PCR assays over McMaster WEC is that they can differentiate strongylid genera present in faeces by using smaller sample volumes and in a shorter time frame than traditional larval cultures. The PCR assays offer the ability to screen individual sheep faecal samples with low worm burdens, making it possible to detect the proportion of a flock which harbour specific strongylid species. In contrast, larval culture differentiations are typically performed on pooled samples to determine the proportion of each species present in an overall strongylid worm population. Larval cultures have also been previously reported to have a biased tendency to identify particular larval species (Dobson et al., 1992). Rapid and accurate identification of strongylid species infections by PCR may be useful for the expeditious diagnosis of highly pathogenic strongylids, such as *H. contortus*, which are capable of causing high mortality rates in susceptible sheep (Dargie and Allonby, 1975). For a true assessment and evaluation of the PCR assays against larval cultures, larval cultures would be necessary for each individual faecal sample.

Another diagnostic method utilised for strongylid species-specific identification is the lectin binding assay that differentiates worm eggs using genus-specific carbohydrates on the surface of eggs (Palmer and McCombe, 1996). This method has proved to be useful for the detection of *H. contortus* and *Trichostrongylus* spp. infections in sheep, with strong
correlations found when compared to those observed in larval culture (Palmer and McCombe, 1996). At present, lectins specific for *T. circumcincta, Oesophagostomum* spp. or *Chabertia ovina*, have not been identified. The time taken to conduct lectin binding assays utilising sugar centrifugation methods (which reduces egg purification time from faeces) (Jurasek *et al.*, 2010) is similar to that of PCR. However, PCR can achieve higher sample throughputs by using 96-well PCR plates and also provide genomic DNA available for the testing of a wider range of pathogens (Bott *et al.*, 2009). More recently, a faecal occult blood assay utilising a commercial “*Haemonchus* Dipstick Test” has been developed. Although having a short processing time (~30 minutes) and capacity to be processed on-farm (rather than in a laboratory), the test is not quantitative and both false positive and negative results have been reported (Colditz and Le Jambre, 2008)

### 5.5 CONCLUSION

The objective of this study was to compare the level of agreement between McMaster WEC and PCR assays (screening genomic DNA extracted directly from faeces utilising a commercial DNA extraction kit) in identifying patent strongyloid infections in lambs. No PCR inhibition was detected in spiked faecal samples and unspiked negative control faecal samples never tested PCR-positive. There was a high level (≥0.93) of agreement between PCR and McMaster WEC test results for identifying strongyloid positive faecal samples. Validation of PCR and WEC results against post-mortem total worm count results, along with further modifications of the individual species-specific PCRs into a single, multiplex, quantitative PCR assay, are both required to facilitate the use of PCR for routine diagnosis of helminthosis in sheep.
CHAPTER 6: STRONGYLID SPECIES EPIDEMIOLOGY IN MEAT LAMBS

MOLECULAR IDENTIFICATION OF NATURALLY ACQUIRED STRONGYLID INFECTIONS IN LAMBS – AN INVESTIGATION INTO HOW LAMB AGE INFLUENCES DIAGNOSTIC SENSITIVITY


6.1 INTRODUCTION

The management and control of strongylid nematodes in commercial sheep enterprises is critical because of the income loss associated with reduced flock productivity (Sackett et al., 2006). The most economically important sheep strongylid genera in southern Australia are Trichostrongylus spp., Teladorsagia circumcincta and Haemonchus contortus. Chabertia ovina, Oesophagostomum spp. and Nematodirus spp. generally contribute to a lesser extent (Besier and Love, 2003). Accurate and reliable diagnosis of strongylid infections is critical for implementing effective control programmes and for monitoring treatment efficacy (Coles et al., 2006; Woodgate and Besier, 2010). Species-specific diagnosis has important implications for rapid identification of highly pathogenic strongylids, approaches to anthelmintic treatment, geographical surveillance of anthelmintic resistance and for understanding strongylid epidemiology in sheep flocks from different geographical regions.

McMaster faecal worm egg counts (WECs) are a useful guide for detection of patent worm burdens in young sheep (Kingsbury, 1965; McKenna, 1981). However this method...
cannot distinguish between different strongylid species contributing to an individual WEC without the use of larval culture differentiation. Larval cultures require an experienced microscopist, are time consuming (1 week), have tendencies to produce biased results and demand relatively large volumes of faecal material and so are typically performed on pooled faecal samples only (Dobson et al., 1992).

There has been recent developments regarding species-specific PCR assays, which are capable of detecting different strongylid worm species in DNA extracted from column-purified worm eggs (Bott et al., 2009). However, the use of such species-specific PCRs to screen genomic DNA extracted directly from faeces has not been well examined, with uneven strongylid worm egg distribution in faecal masses (Sinniah, 1982; Tarazona, 1986; Morgan et al., 2005) a concern for an accurate diagnosis. In addition other challenges with this technique include the small subsample volume of faeces utilised in DNA extractions, the presence of faecal inhibitors (e.g. humic acids and polysaccharides) in samples collected from sheep of different ages and the inability to accurate quantify diagnostic results (Hunt, 2011). Despite these limitations, PCR has advantages over traditional larval culture analysis, including a more rapid and reliable diagnostic identification, an unbiased species identification and the ability to detect different strongylid species. PCR also provides rapid diagnosis of highly pathogenic strongylid species (such as *H. contortus*) and identification of strongylid species, both pre- and post-anthelmintic treatment for efficacy and resistance studies.

A recent study found an overall high level of agreement between PCR and WEC results for the detection of naturally acquired strongylid infections (93%), with DNA extracted directly from faeces (Chapter Five). However, this study had the following
limitations; it was conducted on only those faecal samples collected from lambs on two separate sampling occasions (separated by ~60 days) and all faecal samplings were collected from lambs on-farm. In addition no investigation was conducted into possible associations/correlations between molecular strongylid species (parasite prevalences) and microscopic WEC results. It has been reported that lambs 1-2 months old, have ~80% of their ingested strongylid third stage larvae (L₃) establish infections, with this percentage declining precipitously as lambs age and acquire immunity to the parasites (Dobson et al., 1990a; Dobson et al., 1990c, b, d). Hence a greater number of sampling occasions would be beneficial to assess whether non-patent sources of DNA (larval tissues passing through the gastrointestinal tract of sheep and expelled in faeces) potentially contribute towards false PCR positive identification. Furthermore, DNA inhibitors are potentially present in lamb faeces, particularly in lairage (livestock holding yards at abattoirs) where time held off feed can increase the concentration of inhibitors in faeces.

6.1.1 AIMS AND HYPOTHESES

Therefore, the aims of this study were to:

1. Conduct DNA extractions (using a commercial DNA isolation kit) on individual faecal samples collected from lambs managed under extensive grazing conditions Investigate strongylid genera demographics using species-specific PCR assays.

2. Determine the minimum amount of genomic DNA extracted directly from faeces, for which specific amplification of the ITS-2 region could be achieved.
3. Assess the sensitivity of species specific PCRs screening faecal DNA extracts for strongylid worms across multiple sampling occasions (on-farm and lairage), to determine if the levels of agreement between McMaster WEC and PCR are influenced by time of season, lamb age (acquired immunity) and worm burdens.

4. Examine whether WEC is associated/correlated with molecular strongylid species results (species prevalences, mixed infections and number of species detected per lamb).

5. Compare the prevalence and distribution of different strongylid genera on- and off-farm and whether molecular detection of specific strongylid species is associated with an increased risk of non-pelleted, loose faeces.

The hypotheses of this experiment were that:

1. All species of strongylid will be detected on both farms.

2. Levels of agreement between WEC and PCR diagnostic results are the same for all five samplings (lambs of different age).

3. Molecular PCR results are correlated with number of species detected per lamb.

4. Highest mixed strongylid species prevalences, along with the highest numbers of strongylid species detected per lamb are associated with highest adjusted WEC results.

5. *Trichostrongylus* spp. positive lambs are at greater risk of having non-pelleted, loose faeces when compared to *Trichostrongylus* spp. negative lambs.
6.2 MATERIALS AND METHODS

6.2.1 STUDY SITES, ANIMALS AND EXPERIMENTAL PROTOCOL

The experiment was approved and supervised by the Murdoch University Animal Ethics Committee ( Permit number R2236/09). The two sheep farms in this study were located in southern Western Australia 200-250 km south east of Perth at Pingelly (Farm A: 1500 Ha; 1350 sheep; 32.55° S, 116.87° E) and Arthur River (Farm B: 1250 Ha; 1750 sheep; 33.28° S, 117.01° E) ~150 km apart. These farms experience a Mediterranean climate, with hot, dry summers, cool, wet winters and a predominantly winter rainfall pattern, with an average annual rainfall of 450-500 mm (Hill et al., 2004; Moeller et al., 2008). Winter stocking rates, based on dry sheep equivalents (DSE)/Ha (McLaren, 1997), were 12 DSE/Ha on Farm A and 10 DSE/Ha on Farm B. A general overview of all lamb samplings and time progression throughout the study is shown in Table 3.1 in Chapter Three: Materials and Methods.

On each farm, Merino x Suffolk meat lambs were born and raised on one annual pasture paddock consisting primarily of annual ryegrass (Lolium spp.) and subterranean clover (Trifolium subterraneum). Water was supplied ad libitum via a creek (Farm A), dam (Farm B) or by troughs filled from either of these water sources. Supplementary feed, ~100 g/head/day (35% lupins and 65% oats) was provided for each lamb flock after weaning.

At 2-6 weeks of age (day 0 of study), 111 and 124 female lambs from Farms A and B, respectively, were randomly selected and identified utilising unique numbers and radiofrequency ear tags. Faeces were collected directly from the rectum of each lamb at five separate samplings from day 0 (2-6 weeks of age) to 7-8 months of age (Table 1). A total of 107 and 119 lambs from Farms A and B, respectively, were sampled at all five samplings.
samplings and overall strongylid prevalences were determined from these lambs. Each flock was mustered from their paddock into nearby yards for faecal sampling, except for the final sampling, which took place in lairage facilities following transportation by road to an abattoir for slaughter. Faecal consistency score (FCS) was recorded on a scale of 1-5 (Greeff and Karlsson, 1997).

Faecal samples were collected from each lamb using fresh latex gloves to prevent cross contamination between samples. All faecal samples were placed in individually labelled, airtight 70 mL containers and transported to the laboratory within 6 h of collection. Faecal samples were stored at 2-4 °C and genomic DNA was extracted within 7 days of collection (Yang et al., 2009; Robertson et al., 2010). Lambs on Farm A were consigned for slaughter in two separate groups, the first on day 199 and the second on day 240 of the study. Lambs from Farm B were consigned for slaughter as a single group on day 188 of the study.
Table 6.1: Faecal sampling occasions, lamb age and day of study.

<table>
<thead>
<tr>
<th>Faecal sampling occasion</th>
<th>Stage in lamb’s life</th>
<th>Lamb age</th>
<th>Farm A</th>
<th>Farm B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sampling 1</td>
<td>Marking</td>
<td>2-6 weeks</td>
<td>Study day</td>
<td>Date</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>July 23\textsuperscript{a} 2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Sampling 2</td>
<td>Pre-weaning I</td>
<td>2 months</td>
<td>39 \textsuperscript{a}</td>
<td>Sept 1\textsuperscript{a} 2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>42</td>
</tr>
<tr>
<td>Sampling 3</td>
<td>Pre-weaning II</td>
<td>3-4 months</td>
<td>80</td>
<td>Oct 13\textsuperscript{a} 2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>73 \textsuperscript{a}</td>
</tr>
<tr>
<td>Sampling 4</td>
<td>Post-weaning</td>
<td>6-7 months</td>
<td>194</td>
<td>Feb 8\textsuperscript{a} 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>181</td>
</tr>
<tr>
<td>Sampling 5</td>
<td>Lairage</td>
<td>7-8 months</td>
<td>199/240</td>
<td>Feb 10\textsuperscript{a}/ 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>188</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Indicates after this sampling occasion that lambs received an anthelmintic treatment.

6.2.2 ANTHELMINTIC TREATMENT

Each lamb was treated with 12 mg abamectin and 6 mg selenium (Virbamec Oral Plus Selenium, Virbac) before weaning on days 39 (Farm A) and 73 (Farm B) (Table 6.1).

6.2.3 GENOMIC DNA EXTRACTION

Polymerase chain reaction positive controls for all five strongylid species were created (Table 6.2). Genomic DNA was extracted from 250-300 mg of each of the 1155 faecal samples that were collected from the identified lambs, using the modified Power Soil DNA Isolation Kit (MO BIO Laboratories, Inc.; 2746 Loker Avenue West; Carlsbad, CA 92010) protocol and stored at -20 °C. In brief, samples were subjected to five freeze-thaw cycles, by which each sample was frozen with liquid nitrogen for 4 minutes and then thawed at 90 °C for 4 minutes. The final elution volume of C6 solution (MO BIO Laboratories) was adjusted to 50 µL to increase the final DNA concentration. DNA extraction positive and negative controls, along with serial dilutions of genomic DNA were generated (Table 6.2).
Genomic DNA was extracted from 250-300 mg of each of the 1155 faecal samples collected from the identified lambs using the modified Power Soil DNA Isolation Kit protocol and stored at -20 °C.
Table 6.2: Stepwise procedures, DNA extraction kits and purpose of the different methods utilised to extract genomic DNA from strongylid L₃ and faecal samples.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Methodology prior to DNA extractions</th>
<th>DNA extraction kit</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR positive controls</td>
<td>T. circumcincta, T. colubriformis, H. contortus, C. ovina and Oesophagostomum venulosum L₃ were collected from larval cultures of fresh sheep faeces after microscopic identification. The different L₃ species were separated into 200 µL suspensions (each containing 100 L₃ of only one strongylid species).</td>
<td>DNeasy Blood and Tissue Kit (Qiagen).</td>
<td>Confirm that PCR assays and conditions achieve successful product amplification.</td>
</tr>
<tr>
<td>DNA extraction negative and positive controls</td>
<td>DNA was extracted from negative DNA extraction control samples; 250-300mg of faeces from a sample that returned three WECs &lt;50 epg; (n = 96) and positive DNA extraction controls; 250-300mg of faeces from a sample that returned three WECs &lt;50 epg and spiked with 100 µL (n = 100 L₃) suspension containing equal proportions of L₃ from all five strongylid species; (n = 96).</td>
<td>Power Soil DNA Isolation Kit (MO BIO Laboratories).</td>
<td>Used to determine whether the PCRs were capable of detecting strongylid DNA, whether cross contamination between samples occurred and if any faecal inhibitors were detected in samples.</td>
</tr>
<tr>
<td>Spike and serially diluted genomic DNA</td>
<td>DNA was extracted using 250-300 mg of faeces from a faecal sample that returned three WECs &lt;50 epg. Prior to the extraction each sample was spiked with 100 µL L₃ suspension (n = 100 L₃) containing whole L₃ from only one strongylid species. DNA concentrations were determined using the NanoDrop ND-1000 Spectrophotometer (Thermo Scientific). Genomic DNA was serially diluted from 10⁵ pg to 10,000, 1000, 100, 50, 10, 5, 2, 1 and 0.1 pg/µL. amplification length for each species-specific PCR.</td>
<td>Power Soil DNA Isolation Kit (MO BIO Laboratories).</td>
<td>To determine the minimum amount of genomic DNA extracted from faeces that would achieve successful PCR amplification.</td>
</tr>
<tr>
<td>Field faecal samples</td>
<td>Genomic DNA was extracted from 250-300 mg for each of the 1155 faecal samples collected from the identified lambs in this study.</td>
<td>Power Soil DNA Isolation Kit (MO BIO Laboratories).</td>
<td>Assess the sensitivity and capability of PCRs detecting Strongylid infections across multiple samplings, to determine the levels of agreement between WEC and PCR.</td>
</tr>
</tbody>
</table>

6.2.4 FAECAL WORM EGG COUNTS

Faecal worm egg counts were performed on 2 g faeces from each sample within 2 days of collection using a modified McMaster technique (Lyndal-Murphy, 1993); each egg counted represented 50 epg. At the time of first sampling (day 0), insufficient quantities of...
faeces were collected from all lambs to perform both DNA extraction and WEC; therefore WEC data were missing for some lambs at this sampling.

6.2.5 PCR AMPLIFICATION

For each DNA extract, single-step, conventional PCRs were performed for each strongylid nematode (T. circumcincta, Trichostrongylus spp., H. contortus and C. ovina), as described by Bott et al., (2009). Individual species-specific forward primers (TEL, TRI, HAE and CHO) designed for the second internal transcribed spacer (ITS-2) of ribosomal DNA and the reverse primer (NC2) located at the 5’ region of the 28S rRNA gene, were used to detect each strongylid species. The primer pair TRI-NC2, was capable of detecting all four major Trichostrongylus spp., including T. colubriformis, T. axei, T. vitrinus and T. rugatus, as well as Oesophagostomum columbianum and O. venulosum (Bott et al., 2009).

For each PCR, a 25 µL reaction mixture contained 1 µL DNA, 1x PCR buffer, 2.5 mM MgCl₂, 0.4 mM each deoxynucleotide triphosphate (dNTP), 0.8 µM each oligonucleotide primer and 0.04 U/µL Taq DNA Polymerase (Kapa Biosystems). The PCR thermocycling conditions were as described by Bott et al., (2009).

For any samples that were McMaster WEC flotation positive (≥50 epg) and PCR negative, five separate aliquots (10 µL) of the sample were spiked with 1 µL purified DNA from each of the five strongylid species (T. circumcincta, Trichostrongylus spp., C. ovina, Oesophagostomum spp. and H. contortus). A 1 µL aliquot from each of the spiked 15 µL mixtures was then re-screened by each strongylid species-specific PCR assay to test for inhibition.
To confirm accurate identification of \( L_3 \) from larval cultures, positive control PCR products were sequenced. PCR products from spiked samples and field samples that were PCR positive and McMaster WEC negative \((n = 5)\) were purified using the UltraClean DNA Purification Kit (MolBio) and sequenced using an ABI Prism Terminator Cycle Sequencing Kit (Applied Biosystems) on an Applied Biosystem 3730 DNA Analyzer. Sequence searches were conducted using BLAST\(^1\) and nucleotide sequences were analysed using Chromas Lite version 2.0\(^2\). Sequences were aligned with reference genotypes to confirm positive identification for *H. contortus*, *T. circumcincta*, *Trichostrongylus axei*, *T. colubriformis*, *C. ovina*, *O. venulosum* or *O. columbianum* (GenBank AJ57746.1, AJ577463.1, AY439026.1, EF427624, AY439021.1, Y10790.1 and AJ006150, respectively) using Clustal W\(^3\).

### 6.2.7 STATISTICAL ANALYSIS

Faecal worm egg counts were adjusted for faecal consistency prior to statistical analysis. FCS was adjusted according to the following equation (Le Jambre et al., 2007):

\[
\text{Adjusted WEC} = \left( \frac{\text{WEC}}{34.21 - 5.15 \times \text{FCS}} \right) \times 29.06
\]

Statistical analyses were performed using SPSS Statistics 17.0 for Windows. The WEC data were categorised as positive (WEC \( \geq 50 \) epg) or negative (no strongylid eggs detected). To assess the level of agreement between the McMaster WEC and PCR tests,

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\(^3\) See: [http://www.clustalw.genome.jp](http://www.clustalw.genome.jp).
Cohen’s Kappa (κ) statistic was calculated at each sampling occasion, overall for each farm (all five samplings combined) and overall for the entire study (both farms combined). Categorical data were analysed to test the level of agreement between WEC and PCR results (positive vs. negative), with differences between PCR against WEC, along with WEC against PCR, both accounted for in calculation of the κ statistic.

Sampling prevalences (including 95% confidence intervals) were calculated using the exact binomial method for individual strongylid species, mixed strongylid infections (lambs PCR positive for at least two or more strongylid species at a sampling) and overall (lambs PCR positive for a strongylid species across all five samplings) (Thrusfield, 2007).

The mean number of strongylids detected per lamb at each sampling was calculated using arithmetic means for each farm. Odds ratio risk analyses with Pearson’s $\chi^2$ test for independence or Fisher’s exact two-sided test for significance were conducted to determine if there were significant associations between PCR detection procedures for different strongylid species and for lamb faeces in a non-pelleted form (FCS $\geq$3). Correlation between adjusted WEC and number of strongylid species detected per lamb was estimated by linear regression using a Pearson correlation two-tailed test for significance.

6.3 RESULTS

6.3.1 PCR INHIBITION AND SPIKE ANALYSES

PCR products were amplified from DNA extraction positive controls (faecal samples with WEC <50 epg spiked with L₃) with product sizes in accordance with those described for each strongylid species by Bott et al., (2009). These PCR products amplified in accordance with the L₃ tissue DNA extracts (PCR positive controls), while no products
were amplified from DNA extraction negative controls (faeces only with WEC <50 epg) for any of the species-specific PCRs. Using spike analysis on different serial dilutions for each strongylid species, the minimum amount of genomic DNA required for successful PCR amplification was 2.0-5.0 pg (Table 6.3).
Table 6.3: Minimum amount of genomic DNA extracted directly from faeces, which specific amplification of the ITS-2 region could be achieved using the individual primer sets shown in parenthesis.

<table>
<thead>
<tr>
<th>Farming property and parasite</th>
<th>Detection limit in pg</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Haemonchus contortus</em> (HAE-NC2)</td>
<td>2.0</td>
</tr>
<tr>
<td><em>Teladorsagia circumcincta</em> (TEL-NC2)</td>
<td>5.0</td>
</tr>
<tr>
<td><em>Trichostrongylus</em> spp. (TRI-NC2)</td>
<td>2.0</td>
</tr>
<tr>
<td><em>Chabertia ovina</em> (CHO-NC2)</td>
<td>2.0</td>
</tr>
<tr>
<td><em>Oesophagostomum</em> spp. (TRI-NC2)</td>
<td>5.0</td>
</tr>
</tbody>
</table>

6.3.2 COMPARISON OF MCMASTER WEC WITH PCR

The levels of agreement between the McMaster faecal WEC and the PCR in identifying patent strongylid infections (κ statistic) are shown in Table 6.4. The overall κ value was 0.95 ± 0.01 standard error).

Fourteen of 1004 samples (1.4%) were McMaster WEC flotation positive (all 50 epg) and PCR negative. Following spiking of these sample extracts with 1 μL purified DNA from each of the five strongylid species, the spiked DNA mixture was screened by each species-specific PCR to test for inhibition. All PCR assays amplified in accordance with L3 tissue DNA extracts, indicating that no faecal inhibition was present.

Five of 1004 samples (0.5%) that were McMaster WEC negative and PCR positive were rescreened and the PCR products were sequenced. The sequenced products of *T. circumcincta* (n = 1), *T. colubriformis* (n = 2) and *C. ovina* (n = 2) were 100% identical with GenBank reference sequences, confirming the initial results.
Table 6.4: Strongylid species prevalences and 95% confidence intervals in meat lambs on two farms in southern WA.

<table>
<thead>
<tr>
<th>Sheep age</th>
<th>Farm A</th>
<th></th>
<th>Farm B</th>
<th></th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-weaning</td>
<td>Post-weaning</td>
<td>Pre-weaning</td>
<td>Post-weaning</td>
<td>Overall</td>
</tr>
<tr>
<td></td>
<td>2-6 weeks</td>
<td>2 months</td>
<td>3-4 months</td>
<td>6-7 months (lairage)</td>
<td>2-6 weeks</td>
</tr>
<tr>
<td>Day of study</td>
<td>0</td>
<td>39a</td>
<td>80</td>
<td>194</td>
<td>199 or 240</td>
</tr>
<tr>
<td>Flock Size</td>
<td>550</td>
<td>550</td>
<td>550</td>
<td>500</td>
<td>350</td>
</tr>
<tr>
<td>Stocking Rate (DSE/ha)</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Identified lambs sampled (n)</td>
<td>111</td>
<td>109</td>
<td>108</td>
<td>109</td>
<td>107</td>
</tr>
<tr>
<td>T. circumcincta prevalence</td>
<td>21.6</td>
<td>67.0</td>
<td>25.9</td>
<td>74.3</td>
<td>81.3</td>
</tr>
<tr>
<td>Trichostrongylus spp. prevalence</td>
<td>10.8</td>
<td>52.3</td>
<td>22.2</td>
<td>71.6</td>
<td>79.4</td>
</tr>
<tr>
<td>C. ovina prevalence</td>
<td>7.2</td>
<td>33.0</td>
<td>17.6</td>
<td>27.5</td>
<td>46.7</td>
</tr>
<tr>
<td>Oesophagostomum spp. prevalence</td>
<td>3.6</td>
<td>7.3</td>
<td>6.5</td>
<td>7.3</td>
<td>11.2</td>
</tr>
<tr>
<td>Mixed strongylid prevalence</td>
<td>34.2</td>
<td>87.2</td>
<td>64.8</td>
<td>93.6</td>
<td>100.0</td>
</tr>
<tr>
<td>Average WEC ± S.E.M. (epg)</td>
<td>29 ± 10a</td>
<td>446 ± 45</td>
<td>73 ± 13</td>
<td>432 ± 42</td>
<td>830 ± 53</td>
</tr>
<tr>
<td>Adjusted average WEC ± S.E.M. (epg)</td>
<td>32 ± 11a</td>
<td>552 ± 59</td>
<td>89 ± 18</td>
<td>609 ± 61</td>
<td>1164 ± 101</td>
</tr>
<tr>
<td>κ statistic a ± S.E.M.</td>
<td>1.00</td>
<td>1.00</td>
<td>0.98</td>
<td>0.89</td>
<td>1.00</td>
</tr>
<tr>
<td>Linear regression correlation r2 (P value) between the number of strongylid species per lamb and adjusted WEC</td>
<td>0.412</td>
<td>0.302</td>
<td>0.162</td>
<td>0.178</td>
<td>0.026</td>
</tr>
</tbody>
</table>

Mixed strongylid prevalence = lamb PCR-positive for two or more strongylid species (Note: Haemonchus contortus was not identified in any lamb faecal samples).

Kappa statistic (κ) = the level of agreement between the PCR assays and McMaster WEC diagnostic tests for identifying patent strongylid nematode infections.

S.E.M. = standard error of the mean

a Indicates that lambs received an anthelmintic treatment after this sampling.

b Not all samples had enough faecal material to conduct WEC.
6.3.3 EPIDEMIOLOGY OF STRONGYLID SPECIES

The overall strongylid prevalence was 97.2% (95% confidence interval, 95% CI, 92.0-99.4) for Farm A and 94.1% (95% CI 88.3-97.6) for Farm B. Teladorsagia circumcincta was the most frequent strongylid species identified in both lamb flocks, followed by Trichostrongylus spp., C. ovina and Oesophagostomum spp. (Table 6.4). Patent H. contortus infections were not identified by PCR at either farm. No strongylid species were associated with an increased risk of non-pelleted faeces (FCS ≥3) in lambs from Farm A. T. circumcincta was the only species associated with an increased risk of non-pelleted faeces on Farm B, where lambs which tested T. circumcincta-positive, were 2.30 (95% CI 1.19-5.20; P = 0.043) and 2.63 (95% CI 1.23-7.14; P = 0.041) times more likely to have non-pelleted faeces than negative lambs at the second and final samplings, respectively.

The number of samplings that lambs tested positive for each strongylid species is presented in Figure 6.1. The arithmetic mean number of strongylid species detected per lamb ranged from 0.43 ± 0.06 (mean ± SE) to 2.19 ± 0.07 for Farm A and from 0.34 ± 0.05 to 1.22 ± 0.08 for Farm B at different times of sampling. Lambs (2-6 weeks of age) at the first sampling had the lowest mean number of strongylid species detected per lamb on both farms. The highest mean number of strongylid species detected per lamb was identified in both flocks, either at the sampling when an anthelmintic treatment was administered (Farm B) or at the final sampling with lambs in lairage awaiting slaughter (Farm A). For Farm A, the mean number of strongylid species detected per lamb at lairage, was significantly different to all other samplings (P <0.001), while, for Farm B, the sampling when an anthelmintic treatment was administered was significantly different to the first and third
samplings ($P < 0.001$). Figure 6.2 shows the proportions of lambs from each farm with the number of strongylid species detected per individual.

Figure 6.1: The number of positive samplings that lambs tested positive for each strongylid species, at the two farms in Western Australia.
6.3.4 MIXED STRONGYLID INFECTIONS

In both flocks, 100% of lambs were PCR positive for two or more strongylid genera at one or more samplings, with mixed strongylid prevalences ranging from 26.6-100.0% (Table 6.4). For both farms, *T. circumcincta* and *Trichostrongylus* spp. were the most frequent mixed strongylid infections.

6.3.5 FAECAL WORM EGG COUNT CORRELATIONS WITH PCR RESULTS

On both farms, the highest individual strongylid species prevalences, mixed strongylid prevalence and mean number of strongylid species detected per lamb coincided with the highest average flock WEC; 1,164 epg (adjusted WEC at the final sampling) for Farm A and 273 epg (adjusted WEC at the third sampling) for Farm B (Table 6.4). The
number of strongylid species detected per lamb had a significant positive correlation with WEC and adjusted WEC ($r^2 0.026-0.591$; Table 6.4).

6.4 DISCUSSION

This study utilised recently described diagnostic PCRs (Bott et al., 2009), which had previously been used to screen genomic DNA extracted directly from faeces (Chapter Five). However, in contrast to the previous study, the sensitivity of such a diagnostic approach was examined over an extended period of 8 months (across five separate sampling occasions), to assess the sensitivity of the previously reported molecular diagnostic procedure in comparison with WEC. Bott et al., (2009) demonstrated that PCRs detected naturally acquired strongylid infections following the separation of worm eggs from faeces by sodium nitrate flotation and column-purification. The present study demonstrated that DNA extractions performed directly on faeces, by the use of a commercial DNA isolation kit, could overcome possible inhibitory elements within faeces (collected from lambs both on-farm and in lairage) that may interfere with PCR amplification. This supported the recent findings of high levels of agreement (93%) between PCR and WEC results (Chapter Five), with the current study highlighting a varying sensitivity range at different samplings ($\kappa$ values from 0.85-1.00). All positive controls amplified at the correct product length and all sequenced PCR products were 100% identical with GenBank reference sequences. Although complete inhibition did not occur, the minimum amount of genomic DNA required for PCR amplification of the ITS-2 region, was higher (2-5 pg) in this study compared to a previous study using egg flotation and column-purification (0.1-2 pg) (Bott et al., 2009). Despite no minimum amount of genomic DNA for PCR amplification recorded in a previous study (Chapter Five) which utilised the same molecular diagnostic procedure as the present study, the minimum DNA detection
limit results suggest that low concentrations of faecal inhibitors were likely to exist following DNA extraction directly from faeces.

The source of strongylid DNA includes eggs that have been produced by mature female worms and passed in the faeces, i.e. established, patent infections. Screening for strongylid DNA extracted directly from faeces by PCR for such purposes has some disadvantages. The first is the inability to distinguish between patent and non-patent sources of strongylid DNA. Strongylid worm/larval tissue segments passed in faeces potentially can be amplified by PCR if the DNA yield is above the minimum detection limit.

At the fourth sampling for Farm A lambs (6-7 months old) and the third sampling for Farm B lambs (3-4 months old), kappa values were at their lowest (89% and 85%, respectively). Furthermore, correlations between PCR and WEC decreased as the lambs aged (except for the final sampling on Farm B). This could be due to an increased probability of false PCR positive identification (non-patent DNA sources), whereby acquired immunity developed by lambs grazing strongylid contaminated pastures, would lead to a decreased establishment of ingested L3 and greater biomass of larval tissues (non-patent DNA) passed in the faeces (Dobson et al., 1990a; Dobson et al., 1990c, b, d). The higher levels of agreement observed in lairage (100% and 93% for Farms A and B, respectively) are potentially attributed to an increased worm egg concentration in faeces, as lambs were held off feed and had limited water access prior to slaughter (Jacobson et al., 2009a).

Nevertheless, with some lambs testing negative for strongylid infections while grazing pastures infested with larvae, the strong correlations between WECs and the number of strongylid species detected per lamb by PCR and the highest strongylid species prevalences coinciding with the highest average flock WECs, all indicate that strongylid eggs are likely to be the main source of DNA produced from faecal extractions.
Commercial DNA isolation kits require only a small sub-sample of faeces to be taken from the centre of each sample. Uneven distribution of strongylid worm eggs within faeces (Sinniah, 1982; Tarazona, 1986; Hoste et al., 2001) provokes uncertainty as to whether this sub-sample (0.25-0.30 g) is representative and consistently able to provide an accurate representation of a faecal sample and to achieve an accurate diagnosis. The high levels of agreement from the two category analyses between the McMaster WECs and PCR tests suggest that, although a lower quantity of faeces was used in the molecular identification method, outcomes were similar to those using the McMaster WEC method.

Post-mortem examinations and total worm counts were not performed in the present study, so the infection status of individual lambs was unknown. False PCR positive identification of patent strongylid infections was reported to be low in a recent study where 100 sheep with a very low risk of strongylid infection risk (housed indoors and treated with anthelmintics) never tested PCR positive (Roeber et al., 2011). The 14 samples in this present study that were McMaster WEC positive and PCR negative, amplified in accordance to the species-specific PCRs when spiked with L₃ DNA. Intermittent shedding of strongylid eggs by small numbers of established female worms, an uneven distribution of strongylid eggs within faecal samples (Sinniah, 1982; Tarazona, 1986; Hoste et al., 2001) or DNA concentrations below the minimum DNA detection limit for PCR (2-5 pg), may have lead to some strongylid infections going undetected. Such findings may also indicate the presence of a strongylid species not screened for by a species-specific PCR assay, which had eggs with an indistinguishable morphology from those of other species. For instance, *Cooperia* spp. occur at low proportions in worm populations in sheep flocks in southern Australia (Anderson, 1972, 1973; Barger and Southcott, 1975a), but screening for this strongylid was not performed in the present study.
The main advantages of screening DNA extracted directly from faeces by PCR compared to larval culture, are an increased speed of diagnostic testing, unbiased detection and the ability to detect low worm burdens in small volumes of faeces from individual animals. Fast, accurate identification of strongylid species present within a flock would be useful for the rapid diagnosis of highly pathogenic strongylids (such as *H. contortus*), which can cause high mortalities in susceptible sheep (Dargie and Allonby, 1975; Abbott et al., 1986). This PCR technique determines the proportion of a flock which harbours specific strongylid species and would be beneficial in providing both a fast, accurate diagnosis for effective strongylid control programmes and also for monitoring treatment efficacy (Besier and Love, 2003; Coles et al., 2006; Woodgate and Besier, 2010). Species-specific diagnosis has important implications for treatment decisions, surveillance of anthelmintic resistance and where a high degree of precision for strongylid species identification is required. In contrast, larval cultures have a biased tendency to identify particular larval species (Dobson et al., 1992).

*Haemonchus contortus* was not identified on either farm, even though all spiked *H. contortus* positive controls were amplified successfully by PCR. Optimum survival of free-living *H. contortus* stages occur where adequate moisture, warm temperatures and green pastures enhance L₃ survival (Besier and Dunsmore, 1993a, b) and hot dry conditions dramatically reduce the survival of *H. contortus* larvae (Dobson and Barnes, 1995). The inland regions of southern Western Australia, where the two farms in this study were located, are characterised by environmental conditions whereby high temperatures and a lack of moisture prevail over summer months. Such conditions are unfavourable for *H. contortus* larvae survival (Besier and Dunsmore, 1993a, b).
The high sensitivity of the molecular diagnostic procedure utilised to detect strongylid infections from lambs of various ages, is potentially superior to microscopy-based methods. However, when costs of running the test are included in decision making, affordable diagnostics are typically preferred (Hunt, 2011). Despite DNA polymerases incurring extra costs, PCRs offer increased sample throughput, decreased labour input and the potential to screen for a variety of livestock pathogens (parasites, bacteria and viruses). The major disadvantage of the molecular approach utilised in this study i.e. the inability to distinguish between patent and non-patent sources of strongylid DNA, is potentially an advantage for the early detection of strongylid parasites which contaminate pastures. The differentiation of DNA origin source would not be critical for such a procedure, although the ability to quantify the level of larvae exposure would be. There is potential for further investigation in early larvae detection on pastures, by use of a molecular procedure.

6.5 CONCLUSION

This study utilised PCR assays to identify different patent strongylid species infections in lambs, using DNA extracted directly from faeces with a commercial DNA isolation kit. The sensitivity of such a diagnostic approach was examined over an extended 8 month period across multiple sampling occasions, with levels of agreement between WEC and PCR results ranging from 85-100%. The DNA extraction positive controls amplified in accordance with L₃ tissue DNA extracts. Serial dilutions revealed the minimum amount of extracted genomic DNA required for successful PCR amplification was 2.0-5.0 pg. The highest strongylid species prevalences, mixed strongylid prevalences and mean number of strongylid species detected per lamb coincided with the highest average flock WECs on both farms. These findings indicate that strongylid eggs are likely to be the main...
source of DNA in faecal DNA extracts. Although this molecular technique offers the potential to identify different strongyloid species infections, further comparisons against total worm eggs counts are required to more accurately determine the sensitivity and specificity of such a procedure, as it is potentially influenced by non-patent larvae tissues in faeces, as lambs acquire immunity to these parasites.
CHAPTER 7: PROTOZOA EPIDEMIOLOGY IN MEAT LAMBS

LONGITUDINAL INVESTIGATION OF PROTOZOA PARASITES IN MEAT LAMB FARMS IN SOUTHERN WA


7.1 INTRODUCTION

The intestinal protozoan parasites, Cryptosporidium and Giardia, have the capacity to infect a wide variety of animals, including humans. There is an increasing interest in the prevalence of these parasites in ruminant livestock industries, due to the potential risk they present with respect to the contamination of human drinking water (Zhou et al., 2003; Goh et al., 2004; McCarthy et al., 2008) and their possible impacts on productivity of infected animals.

Several studies in sheep have determined point prevalence of these protozoa at a single time period by sampling a random selection of lambs or sheep within a flock at a specific time (Geurden et al., 2008b; Gómez-Muñoz et al., 2009; Yang et al., 2009; Nolan et al., 2010; Robertson et al., 2010; Wang et al., 2010b), in diarrhoeic lambs (Aloisio et al., 2006) and sheep in lairage (livestock holding facilities before slaughter) (Ryan et al., 2005). However, determination of prevalence at one sampling may not provide a true indication of the overall prevalence in flocks over an extended period of time. Reported sporadic...
(oo)cyst excretion by these protozoa, requires multiple faecal samples to be collected for an accurate diagnosis (O'Handley et al., 1999; Thompson et al., 2008), therefore overall prevalence is potentially underestimated with only one sampling.

Despite this knowledge of sporadic (oo)cyst excretion, there has been little research into overall protozoa prevalences and varying species/genotypes demographics in individual sheep over an extended time period. Santín et al., (2007) sampled 2–6 year old ewes (n=32) and their lambs (n=31) over one month in a barn environment. Robertson et al., (2010) described individual prevalences and different species/genotypes of these protozoa isolated from randomly sampled lambs, from 6 Norwegian sheep flocks at two samplings. However, there is no information available regarding Cryptosporidium and Giardia overall prevalences (tracking individually identified lambs across more than two separate samplings) in young lambs reared under extensive, broad-acre grazing conditions (agriculture practiced on a large scale, whereby extensive areas of land are used for the production of crops or the grazing of livestock at low stocking densities for meat, wool or milk production, with animals not housed indoors).

The aim of this longitudinal study was to investigate the overall prevalences and variation in species/genotype demographics of Cryptosporidium and Giardia in pregnant Merino ewes and their cross-bred lambs. These parasites were detected by screening individual faecal DNA extracts from ewe and lamb faecal samples using PCR. All sheep and lambs were managed under extensive, broad-acre grazing conditions at two separate commercial sheep enterprises in southern Western Australia.

7.1.1 AIMS AND HYPOTHESES

The aims of this longitudinal study were to:
1. Use PCR and qPCR diagnostics to investigate the prevalence and importance on *Cryptosporidium* and *Giardia* in pregnant Merino ewes and meat lambs on-farm and meat lambs in lairage before slaughter at commercial abattoirs.

2. Identify the various species/genotypes of *Cryptosporidium* and *Giardia* in pregnant Merino ewes and meat lambs to determine the different species/genotype demographics.

3. Determine if any meat lamb flocks are a potential zoonotic risk.

4. Compare protozoan prevalences from lambs of different age on-farm and examine if on-farm protozoan prevalences are significantly different to prevalences in lairage.

The hypotheses of this experiment were that:

1. *Cryptosporidium* and *Giardia* prevalences are higher for lambs in a lairage environment compared to on-farm.

2. *Cryptosporidium* and *Giardia* infections are more prevalent in young lambs (2 months of age or younger), when compared to older lambs (greater than 2 months of age).

3. Zoonotic species/genotypes of *Cryptosporidium* and *Giardia* are most prevalent in young lambs.

### 7.2 MATERIALS AND METHODS

#### 7.2.1 STUDY SITES, ANIMALS AND EXPERIMENTAL PROTOCOL

This experiment was approved and supervised by the Murdoch University Animal Ethics Committee (permit R2236/09). The two sheep farms in this study were located at Pingelly (Farm A) and Arthur River (Farm B), both approximately 200–250km south-east of
Perth and separated from one another by approximately 150km. These sheep farms are located in southern Western Australia and experience a Mediterranean environment (hot, dry summers and cool, wet winters) with a predominantly winter rainfall pattern (Hill et al., 2004; Moeller et al., 2008). Both farms have similar average annual rainfall, ranging between 450–500mm, with winter stocking rates measured using dry sheep equivalents per hectare (DSE/ha) (McLaren, 1997). A general overview of all lamb samplings and time progression throughout the study is detailed in Chapter Six.

On each farming property, flocks of 2–4 year old Merino ewes were joined with Suffolk rams in January/February 2009. On each of the two farms, 48 faecal samples were collected from the same pregnant ewes on two occasions, firstly within four months of the commencement of lambing and secondly within two weeks of the commencement of lambing (total 192 samples; 96 from each farm).

When lambs were approximately 2–6 weeks old (day 0 of study), 111 female lambs from Farm A and 124 female lambs from Farm B were randomly selected and identified with a uniquely numbered ear tag and a radio-frequency ear tag. Faeces were collected directly from the rectum of only these identified female lambs, such that faecal samples were collected from the same lambs at five separate samplings, between the first (2–6 weeks of age) and final (7–8 months of age) samplings (Table 2). A total 107 and 119 lambs from Farm A and B respectively, were sampled at all five samplings and overall parasite prevalences were determined from only these lambs. Each flock was mustered from their paddock into nearby yards for faecal sampling, except for the final sampling, which took place in lairage following the transport of lambs by road to the abattoir from their respective farms.
Faecal samples were collected from only the identified lambs in each flock using fresh latex gloves to prevent cross contamination between faecal samples. All faecal samples were placed in individually labelled, airtight 70mL containers and transported to the laboratory within six hours of collection. Faecal samples were stored at 2–4°C and genomic DNA was extracted from each sample within seven days of collection. The transport and storage practices utilised in this study were consistent with other similar studies that used PCR to detect these parasites (Yang et al., 2009; Ng et al., 2010b; Robertson et al., 2010). Lambs on Farm A were consigned for slaughter in two separate groups, the first on day 199 and the second group on day 240 of the study. Lambs from Farm B were consigned for slaughter as a single group on day 188 of the study.

On each farm, lambs were born and raised on one annual pasture paddock consisting primarily of annual rye-grass (Lolium spp.) and subterraneum clover (Trifolium subterraneum). Water was supplied ad libitum via a creek (Farm A), dam (Farm B) or by troughs filled from one of these water sources. Approximately 100g/head/day of a supplementary feed grain mixture (comprising of 35% lupins and 65% oats) was given to each lamb flock after they had been weaned. On each farm lambs were treated with 12mg Abamectin and 6mg selenium (Virbamec Oral Plus Selenium, Virbac Australia) before weaning, on days 39 and 73 of the study for Farm A and B respectively (Table 2 and 3).

7.2.2 GENOMIC DNA EXTRACTION

A total of 1,347 faecal samples (192 faecal samples from pregnant ewes and 1,155 rectal faecal samples from tagged identified lambs) were collected. Genomic DNA was extracted from 250–300mg of each faecal sample using a Power Soil DNA Kit (MolBio, West Carlsbad, California, USA). Minor modifications to the manufacturer’s protocol were
made. Briefly, samples were subjected to five cycles of freeze-thaw (freezing each sample with liquid nitrogen for 4 minutes and then thawing samples at 90°C for 4 minutes). The final elution volume of C6 solution (MolBio) was adjusted to 50µL from the recommended 100µL, in order to increase the final DNA concentration. After elution, DNA was stored at –20°C until use. A negative control (no faecal sample) and positive control (faecal sample spiked with Cryptosporidium and Giardia (oo)cysts) were used in each faecal extraction group.

A 10L water sample was collected from each flock’s drinking water source on the second and third (pre-weaning I and II) samplings. Flock A drank water from a flowing creek with water soaks constructed to trap water (Figure 7.1), while Flock B drank from a clay dam (Figure 7.2). Using Envirochek filters (Pall Life Sciences, New South Wales, Australia), filtration of the water samples was performed in accordance with the manufacturer’s instructions, except for some slight modifications as described by Wohlsen et al., (2004). The eluate was subjected to the same genomic DNA extraction method detailed above.
Figure 7.1: Farm A meat lamb flock river/creek water source.

Figure 7.2: Top – Farm B meat lamb flock dam water source during winter. Bottom – Same water summer.

CHAPTER 7: PROTOZOA EPIDEMIOLOGY IN MEAT LAMBS
7.2.3 PCR AMPLIFICATION

A spike analysis was performed for all PCRs detailed below, in order to determine if there were any PCR inhibitors present in the faecal DNA extracts, which would prevent accurate detection of the protozoan parasites. This was conducted by spiking PCR reactions with 0.5µl of the respective positive controls, with no inhibition detected in any of the samples screened by PCR in this present study. All faecal and genomic livestock water DNA extracts were screened for Cryptosporidium and Giardia, with positive samples sequenced to determine species/genotypes present.

For Cryptosporidium, all faecal and livestock water DNA extracts were screened at the 18S rRNA locus for Cryptosporidium and positives were genotyped by sequencing. A two-step nested PCR protocol was used to amplify the 18S rRNA locus of Cryptosporidium previously described by Ryan et al., (2003), producing a product of ~540bp. All Cryptosporidium positive samples at the 18S rRNA locus were confirmed by another two-step nested PCR protocol, conducted to amplify a product of ~830bp at the actin gene of Cryptosporidium, as described by Ng et al., (2006). This identified if mixed infections existed (i.e. one species amplified at the 18S locus and a different species identified at the actin locus). To confirm and sub-genotype C. parvum positives detected at both the 18S rRNA and actin genes, a two-step nested PCR was at the 60kDa glycoprotein (gp60) gene, which amplified a fragment of ~832bp (Strong et al., 2000; Sulaiman et al., 2005).

All samples positive for C. parvum only (positive for C. parvum at both the 18S rRNA and actin genes) or C. parvum as part of a mixed infection (e.g. C. xiaoii sequenced at 18S rRNA locus and C. parvum at actin locus) were further screened using a C. parvum qPCR
at a unique Cryptosporidium specific protein coding locus, previously described by Yang et al., (2009), to confirm that C. parvum was present in each of the samples.

All samples were screened for Giardia at the gdh (glutamate dehydrogenase) gene as previously described by Read et al., (2004), producing a product of ~480bp. All samples which tested positive for Giardia at the gdh gene, were also screened at the triosephosphate isomerase (tpi) gene with a two-step nested PCR protocol. The primary PCR was performed as described by Sulaiman et al., (2003). For the second round reaction, assemblage-specific primers and conditions for assemblage A (product ~332bp) and E (product ~388bp) were used as previously described (Geurden et al., 2008a; 2009). Each of the positive samples at gdh gene were screened for both G. duodenalis assemblage E and assemblage A to confirm the assemblage detected at the gdh gene and secondly to determine if there were mixed G. duodenalis assemblage infections present (i.e. samples positive for different assemblages at the two loci).

7.2.4 SEQUENCE AND PHYLOGENETIC ANALYSIS

Positive Cryptosporidium (18S rRNA, actin and gp60) and Giardia (gdh) PCR products isolated were purified using an UltraClean™ DNA Purification Kit (MolBio, West Carlsbad, California, USA) and sequenced using an ABI Prism™ Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) on an Applied Biosystem 3730 DNA Analyzer. Sequence searches were conducted using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and nucleotide sequences were analysed using Chromas lite version 2.0 (http://www.technelysium.com.au) and aligned with reference genotypes from GenBank using Clustal W (http://www.clustalw.genome.jp). Phylogenetic trees were constructed for Cryptosporidium isolates at 18S rRNA and actin loci, with
additional isolates obtained from GenBank. Distance estimation was performed, based on evolutionary distances calculation with the Kimura 2-parameter model and grouped firstly using TREECON software (Van de Peer and De Wachter, 1994) to conduct Neighbour-Joining analysis and secondly using Mega 5 software (Kumar et al., 2008) to conduct maximum-parsimony analysis. The confidence of groupings from both analyses was assessed by bootstrapping, using 1000 replicates. A percentage bootstrap support of >50% was used for each phylogenetic tree (Figures 1 and 2).

The nucleotide sequences of sheep genotype I 18S rRNA and actin sequences have been deposited in GenBank under the accession numbers HQ317903 and HQ317904 respectively.

7.2.5 STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS Statistics 17.0 (Statistical Package for the Social Sciences) for Windows (SPSS inc. Chicago, USA). Overall and individual sampling protozoan prevalences (including 95% confidence intervals) were calculated. McNemars chi squared test for two-sided significance was used to determine if significant differences existed between parasite prevalences within each farming property at different samplings, between both farms on the same sampling occasion, between different ages of lambs and between lambs on-farm and in lairage.
7.3 RESULTS

7.3.1 CRYPTOSPORIDIUM PREVALENCE

*Cryptosporidium* was detected on both farms at all samplings, both in the pregnant ewes (Table 7.1) and their lambs (Tables 7.2 and 7.3). From all five samplings, the number of times each lamb tested positive for *Cryptosporidium* ranged from 0–5 (Table 7.2).

**Table 7.1:** Protozoan prevalence and 95% confidence intervals detected by PCR for pregnant ewes on two farms in WA.

<table>
<thead>
<tr>
<th></th>
<th>Farm A</th>
<th>Farm B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 months</td>
<td>2 weeks</td>
</tr>
<tr>
<td>Time before lambing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of pregnant ewes sampled</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>Flock Size</td>
<td>300</td>
<td>270</td>
</tr>
<tr>
<td><em>Cryptosporidium</em> flock prevalence (^a)</td>
<td>6.3</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>(1.3, 17.2)</td>
<td>(2.3, 20.0)</td>
</tr>
<tr>
<td><em>Giardia</em> flock prevalence (^b)</td>
<td>8.3</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>(2.3, 20.0)</td>
<td>(0.5, 14.3)</td>
</tr>
</tbody>
</table>

\(^a\) All *Cryptosporidium* positives were *Cryptosporidium xiaoii*.

\(^b\) All *Giardia* positives were assemblage E.
Table 7.2: The number of samplings that individually identified meat lambs tested positive to *Cryptosporidium*, *Giardia* or both these parasites, at each of the two farms in WA.

<table>
<thead>
<tr>
<th>Farming property and parasite</th>
<th>Number of samplings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Lambs (n)</td>
<td></td>
</tr>
<tr>
<td><em>Cryptosporidium</em></td>
<td></td>
</tr>
<tr>
<td>Farm A</td>
<td>20</td>
</tr>
<tr>
<td>Farm B</td>
<td>33</td>
</tr>
<tr>
<td><em>Giardia</em></td>
<td></td>
</tr>
<tr>
<td>Farm A</td>
<td>36</td>
</tr>
<tr>
<td>Farm B</td>
<td>45</td>
</tr>
<tr>
<td><em>Cryptosporidium and Giardia</em></td>
<td></td>
</tr>
<tr>
<td>Farm A</td>
<td>64</td>
</tr>
<tr>
<td>Farm B</td>
<td>78</td>
</tr>
</tbody>
</table>

Note: A total of 107 and 119 lambs were sampled at all five occasions for Farm A and B respectively.
Table 7.3: Protozoan prevalence and 95% confidence intervals detected by PCR from meat lambs on two farms in WA.

<table>
<thead>
<tr>
<th>Sheep age</th>
<th>Farm A</th>
<th>Farm B</th>
<th>Overall Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-weaning</td>
<td>Post-weaning</td>
<td>Pre-weaning</td>
</tr>
<tr>
<td></td>
<td>2-6 weeks 2 months 3-4 months</td>
<td>6-7 months 7-9 months</td>
<td>2-6 weeks 2 months</td>
</tr>
<tr>
<td></td>
<td>0 39a 80</td>
<td>194 199 or 240</td>
<td>0 42 73a</td>
</tr>
<tr>
<td>Flock Size</td>
<td>550 550 550</td>
<td>500 350</td>
<td>1350 1350 1350</td>
</tr>
<tr>
<td>Stocking Rate (DSE/ha)</td>
<td>12 12 12</td>
<td>10 10</td>
<td>10 9 9</td>
</tr>
<tr>
<td>Identified lambs sampled (n)</td>
<td>111 109 108</td>
<td>109 107</td>
<td>124 123 123</td>
</tr>
<tr>
<td>Cryptosporidium prevalence</td>
<td>31.5 36.7 42.6</td>
<td>26.6 36.4</td>
<td>81.3 18.5 29.2</td>
</tr>
<tr>
<td></td>
<td>(23.0, 41.0)</td>
<td>(18.6, 35.9)</td>
<td>(72.6, 88.2)</td>
</tr>
<tr>
<td>Giardia prevalence</td>
<td>21.6 26.6 27.8</td>
<td>25.7 29.9</td>
<td>67.3 23.4 20.3</td>
</tr>
<tr>
<td></td>
<td>(14.4, 30.4)</td>
<td>(17.8, 34.9)</td>
<td>(57.5, 76.0)</td>
</tr>
<tr>
<td>Cryptosporidium and Giardia prevalence</td>
<td>9.0 12.8 15.7</td>
<td>8.3 14.0</td>
<td>40.2 6.5 5.7</td>
</tr>
<tr>
<td></td>
<td>(4.4, 15.9)</td>
<td>(3.8, 15.1)</td>
<td>(30.8, 50.1)</td>
</tr>
<tr>
<td>Eimeria prevalence</td>
<td>14.9a 16.5 38.0</td>
<td>14.7 12.2</td>
<td>28.3 17.1 19.5</td>
</tr>
<tr>
<td></td>
<td>(6.2, 28.3)</td>
<td>(6.6, 19.9)</td>
<td>(24.3, 32.6)</td>
</tr>
</tbody>
</table>

Note: Overall prevalence is the percentage of lambs that were positive for protozoan parasites at any of the five samplings.

a Indicates after this sampling occasion that lambs received an anthelmintic treatment.
Cryptosporidium prevalence in the ewes 4 months and 2 weeks before lambing commenced, was the same on Farm A and B (Table 7.1). For both farms, those lambs that were faecal sampled on all five sampling occasions, the overall prevalence for Cryptosporidium was 81.3% (87/107) for Farm A and 71.4% (85/119) for Farm B, with lambs classified positive for Cryptosporidium, when at least one positive result was identified for this parasite out of the five samplings (Table 7.3).

The prevalences of Cryptosporidium in the lambs at each of the five samplings on Farm A ranged from a low of 26.6% at the fourth (post-weaning) sampling, to a high of 42.6% at the third (pre-weaning II) sampling (Table 7.3). Highest Cryptosporidium prevalences were found in unweaned lambs between 2–4 months old. Prevalence of Cryptosporidium decreased between the third (pre-weaning II) sampling (42.6%) and the fourth (post-weaning) sampling (26.6%) (P=0.014). Cryptosporidium prevalence was found to have increased between the fourth (post-weaning) sampling of 26.6% and the final (lairage) sampling of 36.4% (P=0.002). No other significant differences between Cryptosporidium prevalences were observed (Table 7.3 and Figure 7.3).

Across the five samplings, Cryptosporidium prevalence in Farm B lambs ranged from a low of 18.5% at the first (marking) sampling, to a high of 42.0% at the final (lairage) sampling (Table 7.3). Cryptosporidium prevalence on Farm B at the first (marking) sampling was 18.5% and this was significantly lower than all subsequent sampling prevalences (P<0.001). A Cryptosporidium prevalence of 29.2% at the second (pre-weaning I) was different to subsequent prevalences of 39.0% at the third (pre-weaning II) sampling (P=0.08) and 42.0% at the final (lairage) sampling (P=0.002) (Table 7.3 and Figure 7.3).
At the first (marking) sampling, there was a difference (P<0.001) between the Cryptosporidium prevalence on Farm A (31.5%) and B (18.5%). This was the only significant difference in Cryptosporidium prevalences between farms, within each sampling.

![Cryptosporidium Prevalence](image)

Figure 7.3: Cryptosporidium prevalence on both farms at each of the five sampling occasions (95% CI indicated by standard error bars).

When comparing the average of the three pre-weaning prevalences to the average of the two post-weaning prevalences for Cryptosporidium, there were no significant differences on either farm (Figure 7.4).

The prevalence of Cryptosporidium in young lambs (≤2 months old) compared to old lambs, was not significantly different for Farm A, while older lambs on Farm B had a significantly higher prevalence than young lambs (P=0.009) (Figure 7.5).
Figure 7.4: Average prevalence of pre- (3 samplings) and post-weaning lambs (2 samplings) positive for Cryptosporidium on each farm (95% CI indicated by standard error bars).

<table>
<thead>
<tr>
<th></th>
<th>Pre-wean</th>
<th>Post-wean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farm A</td>
<td>40.3</td>
<td>34</td>
</tr>
<tr>
<td>Farm B</td>
<td>28.9</td>
<td>37.8</td>
</tr>
</tbody>
</table>

Figure 7.5: Average percentage prevalence of young lambs (first two samplings; lambs two months of age and younger) and old lambs (final three samplings) positive for Cryptosporidium on each farm (95% CI indicated by standard error bars).

<table>
<thead>
<tr>
<th></th>
<th>Young lambs</th>
<th>Old lambs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farm A</td>
<td>34.1</td>
<td>35.2</td>
</tr>
<tr>
<td>Farm B</td>
<td>23.9</td>
<td>38.2</td>
</tr>
</tbody>
</table>
For Farm B, the final lairage sampling *Cryptosporidium* prevalence was higher than the combined average of the prevalences from the first four sampling occasions on-farm (P=0.038) (Figure 7.6).

![Graph showing Cryptosporidium prevalence](image)

**Figure 7.6:** Average percentage prevalence of lambs on-farm (4 samplings) and lairage (1 sampling) positive for *Cryptosporidium* on each farm (95% CI indicated by standard error bars).

### 7.3.2 *CRYPTOSPORIDIUM* SPECIES AND GENOTYPES

The *Cryptosporidium* genotypes identified are shown in Tables 7.4 and 7.5, along with Figure 7.7. A combined total of 387 isolates were identified as positive for *Cryptosporidium* at the 18S rRNA and actin loci. From the pregnant ewes on both farms, all isolates were identified as *C. xiaoi* at the 18S rRNA locus and this was confirmed at the actin locus.

On Farm A, *C. xiaoi* was the most common *Cryptosporidium* species isolated from lambs with 134/189 (70.9%) positive isolates. At each of the last three samplings, the
proportion of *Cryptosporidium* positive isolates identified as *C. xiao* was 78.3% (36/46), 75.9% (22/29) and 82.0% (32/39) respectively (Table 7.4).

*Cryptosporidium ubiquitum* was most commonly isolated in young, unweaned lambs on Farm A at the first two samplings. At the first (marking) and second (pre-weaning I) samplings, 37.1% (13/35) and 17.5% (7/40) of *Cryptosporidium* positive isolates were identified as *C. ubiquitum*, respectively. *Cryptosporidium ubiquitum* was also detected at each of the last three samplings, but only in 1–2 isolates at each sampling (Table 7.4).

*Cryptosporidium parvum* was identified in a total of 18 isolates from Farm A lambs across the five samplings, which were sub-genotyped at the gp60 locus and identified as the *C. parvum* genotype IIdA20G1. The highest number of *C. parvum* isolates was six and this was identified at the third (pre-weaning II) sampling, although the highest proportion of *Cryptosporidium* positive isolates identified as *C. parvum*, was 13.8% (4/29) at the fourth (post-weaning) sampling.

A total of 12 mixed *Cryptosporidium* species infections of *C. xiao* and *C. parvum* were identified in lambs from Farm A, whereby *C. xiao* was isolated at the 18S rRNA locus and *C. parvum* isolated at the actin locus. This was confirmed by qPCR at the diagnostic locus for *C. parvum*. Four mixed *Cryptosporidium* species infections were identified at the first (marking) sampling and two identified at each of the other four samplings. In addition, there was one mixed *Cryptosporidium* species infection of *C. ubiquitum* and *C. parvum* identified at the first (marking) sampling (Table 7.4).

Farm B had similar species/genotype demographics to Farm A. *Cryptosporidium xiao* was the most common species detected in lambs and was detected in 76.3% (151/198) of the *Cryptosporidium* positive isolates. *Cryptosporidium xiao* was the most
common species isolated in older lambs (3–7 months of age) with 77.1% (37/48), 82.9% (34/41) and 84.0% (42/50) of *Cryptosporidium* positive isolates recorded for the last three samplings respectively (Table 7.4).

*Cryptosporidium ubiquitum* was most commonly isolated in young lambs from Farm B, identified in 13.0% (3/23) and 19.4% (7/36) of *Cryptosporidium* positive isolates at the first two samplings. *Cryptosporidium ubiquitum* was identified at the last three samplings but at low proportions (only 1 to 2 isolates per sampling). *Cryptosporidium parvum* was detected only at the last two samplings (post weaning and lairage) (Table 7.4).

A novel *Cryptosporidium* genotype, hereafter referred to as sheep genotype I, was identified in six isolates from Farm B, all of which were genetically distinct at both the 18S and actin loci. At the 18S locus, multiple mutations were identified at positions 150, 153, 181, 197, 308, 311, 345, 359, 361, 365 – 368, 390 and 392. There were also significant deletions in the sheep genotype I isolates when compared to *C. ubiquitum* calf and goat sequences, which occurred between positions 380 – 389 and 394 – 417. At the actin locus, base pair differences were detected at positions 250, 253, 279 – 283 and 87 – 88. There was also one T insertion at bp position 255 in the novel genotype isolates. This resulted in sheep genotype I having 13 different amino acids coded at the end of the actin sequence (250 – 289bp), when compared to *C. ubiquitum* goat and calf isolates. Partial sequences at the 18S rRNA and actin genes of this genotype are detailed in the thesis Appendix (Chapter Thirteen).

Mixed *Cryptosporidium* species infections of *C. xiaoi* and *C. parvum* were identified in lambs from Farm B at all five samplings, with the highest proportion of *Cryptosporidium* positive isolates being 21.7% (5/23) and detected at the first (marking) sampling.
Two, one and one isolates of *C. andersoni* were identified at the 18S rRNA and actin loci respectively at the third (pre-weaning II), fourth (post-weaning) and final (lairage) samplings for Farm B. On Farm A, all creek water samples tested negative for *Cryptosporidium*, however both dam water samples collected from Farm B tested positive for *C. xiaoii* at the 18S rRNA and actin loci.

The different *Cryptosporidium* demographics in the pre- (3 samplings) and post-weaning (2 samplings) lambs from both of the farm results are presented in Figure 7.8, with *C. xiaoii* the most common in both pre- and post-weaning lambs. *Cryptosporidium ubiquitum* was most common in pre-weaned lambs, with *C. parvum* most common in post-weaning lambs. Similar results were observed when species/genotypes isolated from young and old lambs were compared (Figure 7.9).
Table 7.4: Number of different species and genotypes of *Cryptosporidium* in growing lambs on two Western Australian farms.

| Sheep age | Farm A | | Farm B | | | | | | | | |  |
|-----------|--------|---|---|---|---|---|---|---|---|---|---|---|---|
|           | Pre-weaning | Post-weaning | 7-9 months (lairage) | TOTAL | Pre-weaning | Post-weaning | 7-9 months (lairage) | TOTAL |
|           | 2-6 weeks | 2 months | 3-4 months | 6-7 months | TOTAL | 2-6 weeks | 2 months | 3-4 months | 6-7 months | TOTAL |
| Day of study | 0 | 39 | 80 | 194 | 199 or 240 | 0 | 42 | 73 | 181 | 188 |
| *Cryptosporidium* | | | | | | | | | | | | | |
| C. parvum only | 0 | 4 | 6 | 4 | 4 | 18 | 0 | 0 | 0 | 0 | 2 | 3 | 5 |
| C. xiaoï only | 134 | 13 | 25 | 37 | 34 | 42 | 151 |
| C. ubiquitum only | 24 | 3 | 7 | 2 | 1 | 0 | 2 | 0 | 2 | 1 | 1 | 14 |
| Sheep genotype I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 1 | 6 |
| C. andersoni only | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 1 | 4 |
| Mixed C. xiaoï + C. parvum | 12 | 5 | 4 | 5 | 2 | 2 | 18 |
| Mixed C. ubiquitum + C. parvum | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total | 35 | 40 | 46 | 29 | 39 | 189 | 23 | 36 | 48 | 41 | 50 | 198 |
Table 7.5: Number of *Cryptosporidium* isolates identified at respective loci from lambs on each farm.

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Farm A</td>
<td>146</td>
<td>24</td>
<td>0</td>
<td>18</td>
<td>134</td>
<td>23</td>
<td>12</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Farm B</td>
<td>169</td>
<td>14</td>
<td>4</td>
<td>5</td>
<td>151</td>
<td>14</td>
<td>18</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>315</td>
<td>44</td>
<td>2</td>
<td>23</td>
<td>285</td>
<td>44</td>
<td>30</td>
<td>1</td>
<td>6</td>
</tr>
</tbody>
</table>
Figure 7.7: Prevalence of the different Cryptosporidium species/genotypes isolated from lambs at each individual sampling occasion for each farm.
Figure 7.8: Prevalence of the different *Cryptosporidium* species/genotypes in pre (first three samplings) and post-weaned (last two samplings) lambs from both farms in southern WA.

Young Lambs

Old Lambs

Figure 7.9: Prevalence of the different *Cryptosporidium* species/genotypes in young (first two samplings) and old (last three samplings) lambs from both farms in southern WA.
7.3.3 **CRYPTOSPORIDIUM PHYLOGENETIC ANALYSIS**

At the 18S locus, phylogenetic analysis confirmed the six sheep genotype I isolates from Farm B, as genetically distinct, sharing only a 94% similarity to calf and goat *C. ubiquitum* sequences, as reported by Nichols *et al.*, (2010) across 420 bp (Figure 7.10). At the actin locus, sheep genotype I shared only a 95% similarity to actin calf and goat *C. ubiquitum* sequences, as reported by Nichols *et al.*, (2010) across 289 base pairs (Figure 7.11).

7.3.4 **GIARDIA PREVALENCE**

*Giardia* was detected in both flocks at all samplings in the ewes (Table 7.1) and lambs (Tables 7.2 and 7.3). Over the five samplings for the lambs, the number of times each lamb tested positive to *Giardia* ranged from 0 – 5 (Tables 7.2).

*Giardia* prevalences in the ewes 4 months and 2 weeks before lambing commenced were the same on Farm A and B (Table 7.1).

On both farms, for lambs that were faecal sampled at all five samplings, the overall prevalence for *Giardia* was 67.3% (72/107) for Farm A and 60.5% (72/119) for Farm B, with lambs classified positive for *Giardia*, having one or more positive test results for the parasite across the five samplings. *Giardia* prevalence in lambs was lower than the *Cryptosporidium* prevalence at all samplings for Farm A and all samplings after the first (marking) sampling for Farm B (Table 7.3).
Figure 7.10: Phylogenetic relationships of *Cryptosporidium* species and genotypes isolated from lambs in southern Western Australia, with some known *Cryptosporidium* species and genotypes, as inferred by Neighbour-joining analysis of Kimura’s distances calculated from pair-wise comparisons of partial (~540bp) 18S rRNA gene sequences. Percentage bootstrap values (>50%) from 1000 pseudoreplicates are shown for both the Neighbour-joining (first value) and maximum-parsimony (second value) analyses. ns = node with bootstrap value <50%.
Figure 7.11: Phylogenetic relationships of Cryptosporidium species and genotypes isolated from lambs in southern Western Australia, with some known Cryptosporidium species and genotypes, as inferred by Neighbour-joining analysis of Kimura’s distances calculated from pair-wise comparisons of partial (~830bp) actin gene sequences. Percentage bootstrap values (>50%) from 1000 pseudoreplicates are shown for both the Neighbour-joining (first value) and maximum-parsimony (second value) analyses. ns = node with bootstrap value <50%.
Giardia prevalence in lambs on Farm A ranged from a low of 21.6% at the first (marking) sampling, to a high of 29.9% at the final (lairage) sampling (Table 7.3 and Figure 7.12). While on Farm B, Giardia prevalence ranged from a low of 20.3% at the second (pre-weaning I) sampling to a high of 29.4% at the final (lairage) sampling. The highest Giardia prevalence was recorded in weaned lambs at the fourth (post weaning) and final (lairage) samplings (Table 7.3). There were no significant differences in Giardia prevalence for Farm A and B lambs between all samplings. Furthermore, there were no differences in Giardia prevalence between the two farms, within each sampling.

![Figure 7.12: Giardia prevalence on both farms at each of the five sampling occasions (95% CI indicated by standard error bars).](image-url)

Figure 7.12: Giardia prevalence on both farms at each of the five sampling occasions (95% CI indicated by standard error bars).
No significant differences were found for each farm when pre- and post-weaning *Giardia* prevalences were compared (Figure 7.13), along with young and old lamb prevalences (Figure 7.14).

![Average prevalence of pre- and post-weaning lambs](image1)

**Figure 7.13:** Average prevalence of pre- (3 samplings) and post-weaning lambs (2 samplings) positive for *Giardia* on each farm (95% CI indicated by standard error bars).

![Average prevalence of young and old lambs](image2)

**Figure 7.14:** Average prevalence of young lambs (2 samplings) and old lambs (3 samplings) positive for *Giardia* on each farm (95% CI indicated by standard error bars).
For both farms, the final (lairage) sampling *Giardia* prevalence at the abattoirs was higher than the combined average of the prevalences from the first four sampling occasions on-farm, although this difference was not significant (Figure 7.15).

![Figure 7.15: Average percentage prevalence of lambs on-farm (4 samplings) and lairage (1 sampling) positive for *Giardia* on each farm (95% CI indicated by standard error bars).](image)

**7.3.5 GIARDIA ASSEMBLAGES**

All 12 positives from pre-lambing ewes on both farms were identified as assemblage E at both the *gdh* and *tpi* loci (Tables 7.6 and Figure 7.16). On Farm A, 90.2% (84.1 – 94.5) of the 143 *Giardia* positive isolates were identified as assemblage E at both *gdh* and *tpi* loci. The highest proportion of assemblage E was identified at the second (pre-weaning I) sampling, which was 96.6% (82.2 – 99.9). All assemblage A isolates identified on both farms belonged to the AI sub-assemblage group. On each farm, assemblage A was identified at a low prevalences (one to four isolates) at each of the five sampling occasions, with the highest prevalence of 12.5% (3.5 – 29.0) found at the final (lairage) sampling on
Farm A. Mixed assemblage A and E infections were identified by screening at the $gdh$ locus and then also at $tpi$ locus (Tables 7.6 and Figure 7.16). Five and seven mixed assemblages A and E infections were identified at all of the last three sampling occasions for both Farm A and B respectively (Tables 7.6 and Table 7.7).

The sheep drinking water sources on both farms (creek and dam water) both tested negative for *Giardia* on both sampling occasions. The different *Giardia* genotype demographics in the pre- (3 samplings) and post-weaning (2 samplings) lambs from both of the farm results are presented in Figure 7.17, with assemblage E the most common in both pre- and post-weaning lambs. Assemblage A and mixed assemblage E and A infections were most common in post-weaning lambs. Similar results were observed when assemblages isolated from young and old lambs were compared (Figure 7.18).
Table 7.6: Number of different assemblages of *Giardia* in growing lambs on two WA farms.

<table>
<thead>
<tr>
<th>Sheep age</th>
<th>Farm A</th>
<th>Farm B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-weaning</td>
<td>Post-weaning</td>
</tr>
<tr>
<td>Sheeps age</td>
<td>2-6 weeks</td>
<td>2-6 months</td>
</tr>
<tr>
<td>Day of study</td>
<td>0</td>
<td>39</td>
</tr>
</tbody>
</table>

*Giardia*

- *G. duodenalis assemblage E* only: 22 | 28 | 27 | 26 | 26 | 129 | 28 | 23 | 28 | 32 | 30 | 141
- *G. duodenalis assemblage A* only: 2 | 1 | 1 | 1 | 4 | 9 | 1 | 1 | 2 | 1 | 2 | 7
- Mixed *G. duodenalis assemblage A + E*: 0 | 0 | 2 | 1 | 2 | 5 | 0 | 1 | 2 | 1 | 3 | 7
- **Total**: 24 | 29 | 30 | 28 | 32 | 143 | 29 | 25 | 32 | 34 | 35 | 155

Table 7.7: Number of *Giardia* isolates identified at respective loci from lambs on each farm.

<table>
<thead>
<tr>
<th>Property</th>
<th>AssE (<em>gdh</em>)</th>
<th>AssA (<em>gdh</em>)</th>
<th>Mixed AssE and AssA (<em>gdh</em>) and (<em>tpi</em>)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farm A</td>
<td>129</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>Farm B</td>
<td>141</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>270</strong></td>
<td><strong>16</strong></td>
<td><strong>12</strong></td>
</tr>
</tbody>
</table>
Figure 7.16: Prevalence of the different *Giardia* assemblages isolated from lambs at each individual sampling occasion for both farms.
**7.3.6 GIARDIA PHYLOGENETIC ANALYSIS**

All assemblage A isolates identified on both farms belonged to the AI sub-assemblage group (Figure 7.19). Mixed *Giardia* genotype infections with assemblage A and E were identified in lambs from Farm B at the final four sampling occasions, while on Farm A, only at the final three sampling occasions. Mixed *Giardia* genotype infections were not found in young lambs on at the first sampling occasion for either farm.
Of the 141 assemblage E isolates discovered on Farm B, 38 of these were grouped with an assemblage E outgroup isolate identified from cattle (Figure 7.19).

**Figure 7.19:** Phylogenetic relationships of *Giardia duodenalis* assemblages isolated from lambs in southern Western Australia, with some known assemblages, as inferred by Neighbour-joining analysis of Kimura’s distances calculated from pair-wise comparisons of partial (~480bp) glutamate dehydrogenase. Percentage bootstrap values (>50%) from 1000 pseudoreplicate are shown for both the Neighbour-joining (first value) and maximum likelihood (second value) analyses. ns = node with bootstrap value <50%.
7.3.7 MIXED INFECTIONS OF CRYPTOSPORIDIUM AND GIARDIA

Over the five samplings, the number of occasions each lamb tested positive for both Cryptosporidium and Giardia ranged from 0 – 3 and 0 – 4 for Farm A and B, respectively (Table 7.2). The Cryptosporidium and Giardia overall prevalence was 40.2% (43/107) for Farm A and 34.5% (41/119) for Farm B (Table 7.3). For both farms at each lamb sampling, there were mixed protozoan infections identified. On Farm A there were no significant differences between the number of mixed and single protozoan infections across sampling occasions. On Farm B, the last three samplings had higher proportions of mixed protozoan infections identified, when compared to the first two samplings (P<0.001).

7.3.8 EIMERIA

Eimeria (coccidia) parasites were detected by microscopy in lambs from both farms at all sampling occasions. For Farm A and B, Eimeria oocysts were detected in 61.7% (66/107) and 68.9% (82/119) of lambs respectively at least once out of the five sampling occasions (Table 7.3). The highest prevalence was observed on both farms at the third (pre-weaning II) sampling occasion, which was 38.0% (28.8 – 47.8) for Farm A and 50.4% (41.2 – 59.5) for Farm B (Figure 7.20). The prevalence of Eimeria at the third (pre-weaning II) sampling was significantly different to all other sampling occasions on both farms (P<0.001).
7.4 DISCUSSION

In the present study, Cryptosporidium and Giardia were both commonly identified in young, meat lambs across all five samplings for both farms. Each farm had over 72% of the identified lambs test positive for Cryptosporidium at least once and over 60% test positive for Giardia at least once. Also 35–40% of the identified lambs had a mixed Cryptosporidium and Giardia infection detected on at least one of the five samplings.

The Cryptosporidium and Giardia sampling prevalences were within similar ranges to those described in previous random sampling studies conducted in the USA (Santin et al., 2007), Europe (Geurden et al., 2008b; Gómez-Muñoz et al., 2009; Robertson et al., 2010) and Australia (Yang et al., 2009; Nolan et al., 2010). However, the overall protozoan prevalences (lambs positive for a parasite at least once out of the five samplings) for Cryptosporidium (ranging between ~71–81%) and Giardia (ranging between ~60–67%) are lower.
were significantly higher than all individual sampling prevalences reported both in this and previous studies. On both farms, some lambs tested positive to Cryptosporidium or Giardia at all five samplings, indicating they were either unable to clear naturally acquired protozoan infections or were repeatedly re-infected from their environment or other flock members. This has been documented in previous studies on Giardia in farm animals (Taylor et al., 1993). The findings of high overall Cryptosporidium or Giardia prevalences and the varying number of samplings at which lambs tested positive to either protozoa (Table 4), highlights the importance of multiple faecal sample testing to accurately identify animals that have contracted either protozoan infection. This is because random sampling at a single time point is likely to underestimate the overall prevalence of these protozoa across an extended time period, due to sporadic (oo)cysts excretion (O'Handley et al., 1999; Thompson et al., 2008), which leads to some infected lambs going undetected.

There appears to be differences in the prevalence of Cryptosporidium and Giardia in different geographical locations, with European studies reporting higher Giardia prevalence relative to Cryptosporidium prevalence (Castro-Hermida et al., 2007; Geurden et al., 2008b). However, studies in the USA and Australia have reported higher Cryptosporidium prevalence relative to Giardia prevalence (Santin et al., 2007; Yang et al., 2009). In the present study, there were significantly higher prevalences of both Cryptosporidium and Giardia in the growing lambs (18.5 – 42.6%), when compared to that of pregnant ewes (<10%). Protozoa prevalences at the final (lairage) sampling may have been higher than prevalences at fourth (post-weaning), due to elevated stress levels associated with the mustering, transport and a lairage environment (Sotiraki et al., 1999).
Four species of *Cryptosporidium* were identified in lambs in this present study; *C. parvum*, *C. xiaoi C. ubiquitum* and *C. andersoni*, while one novel genotype (sheep genotype I) was also detected. *Cryptosporidium xiaoi* was the most common species isolated from lambs on both farms and the only species identified in the pregnant ewes. Previous Australian studies have identified *C. parvum*, *C. hominis*, *C. xiaoi*, *C. ubiquitum*, *C. andersoni*, pig genotype II, *C. fayeri* and *C. suis* in sheep or lambs (Ryan *et al.*, 2005; Yang *et al.*, 2009). Previous studies in the UK have reported that *C. parvum* was the most common *Cryptosporidium* species identified in unweaned lambs (Pritchard *et al.*, 2007; Mueller-Doblies *et al.*, 2008). A previous Australian study also reported a high proportion of *C. parvum* isolates (53/115) in unweaned lambs from farms in southern Western Australia (Yang *et al.*, 2009). However, in the present study, *C. parvum* was identified at low proportions in lambs from the two farms and was commonly identified as part of *C. xiaoi* and *C. parvum* mixed infection (Table 6). All *C. parvum* isolates isolated at the *gp60* locus were *C. parvum* genotype IIdA20G1, and this is the first known record of this *C. parvum* genotype isolated from either sheep or lambs. Mixed infections of *Cryptosporidium* spp. were identified at all samplings at both farms, with the highest proportions recorded at the third (pre-weaning II) and final (lairage) samplings.

Recently, *C. ubiquitum* (previously known as the cervine genotype) was described as a valid *Cryptosporidium* species (Fayer *et al.*, 2010b) and although identified in humans worldwide (Ong *et al.*, 2002; Learmonth *et al.*, 2004; Chalmers *et al.*, 2009), this species has not been detected in any human cryptosporidiosis cases in Western Australia to date (Ng *et al.*, 2010a; 2010b). *Cryptosporidium ubiquitum* was found in high proportions in young lambs up to 2 months old on both farms, although the species was still detected in older lambs. *Cryptosporidium ubiquitum* is one of the common *Cryptosporidium* species
isolated from sheep, with a study of five sheep farms in China reporting *C. ubiquitum* as the major *Cryptosporidium* species present in lambs and sheep (Wang *et al.*, 2010b). *Cryptosporidium andersoni* was isolated from only Farm B lambs 3 months of age and older, with previous studies in Western Australia (Yang *et al.*, 2009) and China (Wang *et al.*, 2010b), also detecting this species at low proportions (<10%). The major host for *C. andersoni* is cattle, with often asymptomatic infections of this parasite reported (Anderson, 1998; Fayer *et al.*, 2005), while sheep act as a minor host with no clinical signs of infection reported (Anderson, 1998; Smith and Nichols, 2010).

A novel genotype (sheep genotype I) was identified in six lambs from Farm B. Phylogenetic analyses at both the 18S rRNA and actin loci, suggests that sheep genotype I is most closely related to *C. ubiquitum* and is likely a distinct species. The range of percent similarities between currently accepted *Cryptosporidium* species at the 18S rRNA locus (89 – 99.8%) and the actin locus (76–98.7%), is one of the criteria used to delimit species within the genus *Cryptosporidium* (Xiao *et al.*, 2004). The range of genetic similarities between sheep genotype I and all other *Cryptosporidium* species are 90–94% at the 18S rRNA locus and 78–95% at the actin locus, which is well within this range criteria. Unfortunately it was not possible to conduct morphological analysis on the sheep genotype I, as fresh faecal samples were no longer available. The placing of sheep genotype I with the intestinal *Cryptosporidium* species, suggests that it may be similar in size to *C. parvum*, although further biological studies are required to confirm the species status of sheep genotype I.

Lambs on Farm B drank water from a dam in which *C. xiaoii* (oocysts) were detected on two samplings. Resistant, thick walled *Cryptosporidium* oocysts are capable of surviving
in favourable cool, damp environmental conditions for extended periods of 3–6 months (Robertson et al., 1992; Fayer et al., 1996; Carey et al., 2004). Phylogenetic analyses at the 18S rRNA and actin loci (Figure 1 and 2) shows that some C. xiaoi isolates from both the lambs and water source were identical to one another. It is possible that the dam on Farm B was contaminated with lamb or sheep faecal material containing C. xiaoi oocysts. No Giardia cysts or trophozoites were detected in any of the water samples on Farm B.

The zoonotic G. duodenalis assemblage A and non-zoonotic assemblage E were identified in lambs on both farms, with all Giardia assemblage A isolates identified at gdh locus as AI sub-assemblage group. To date, studies in sheep have reported predominantly assemblage E, with lower proportions of assemblage A and also assemblage B (Ryan et al., 2005; Aloisio et al., 2006; Santin et al., 2007; Gómez-Muñoz et al., 2009; Yang et al., 2009; Lebbad et al., 2010). In a recent study in Australia, lambs (of an unknown age) randomly selected from 3 farms had Giardia prevalences ranging from 4.3 – 23.7% and that assemblage A was the most common genotype isolated (Nolan et al., 2010). Other recent studies in Australia (Ryan et al., 2005; Yang et al., 2009), Spain (Gómez-Muñoz et al., 2009), Belgium (Geurden et al., 2008b), Norway (Robertson et al., 2010) and the USA (Santin et al., 2007) reported that assemblage E was most common in lambs and sheep. In the present study, assemblage E was most commonly identified at all samplings, with assemblage A more common in post-weaning lambs, which has been reported in previous Australian sheep studies (Ryan et al., 2005; Yang et al., 2009). As positive samples in this study were screened at two loci (gdh and tpi), mixed assemblage A and E infections were able to be identified, with totals of five and seven mixed infections found on Farm A and B, respectively. These mixed infections were most common in lambs 3 months of age and

CHAPTER 7: PROTOZOA EPIDEMIOLOGY IN MEAT LAMBS
older, with a recent study in Sweden also detecting mixed assemblage A and E infections in 3/26 ~1–3 month old lambs, at the gdh, tpi and β-giardin loci (Lebbad et al., 2010).

The highest *Eimeria* spp. prevalence of 38.0% and 50.4% (determined by microscopy) for Farms A and B respectively, was in lambs of 3 – 4 months of age. After weaning, the prevalence fell to approximately 12 – 14% on both farms. Prevalences of 30% and greater have also been observed in lambs in Turkey, with lambs of approximately 1 and 2 months of age having *Eimeria* prevalences of 50.0% and 76.8% (Ozdal et al., 2009).

### 7.4 CONCLUSION

This study found high overall prevalences of *Cryptosporidium* and *Giardia* in lambs extensively grazed on two sheep farms in southern Western Australia, across five separate samplings. Changing protozoan species/genotype demographics was found in lambs of different ages and low proportions of isolates were identified as zoonotic. Zoonotic *Cryptosporidium* and *Giardia* isolates were most commonly found as part of a mixed species/genotype infection in older lambs. In this study, over 60% of identified lambs naturally acquired a *Cryptosporidium* or *Giardia* infection at least once on each farm. The effect that these protozoan infections have on lamb meat production is unclear, with only incidences of diarrhoea documented in previous literature. If future studies identify associations of production losses with these protozoa, then further investigation into their common routes of infection should be determined. This would benefit the construction and development of future control practices that can be utilised by extensive grazing sheep enterprises, to limit the transmission of these parasites between animals and their environment.
CHAPTER 8: INTERNAL PARASITES ASSOCIATED WITH PRODUCTIVITY

IMPACTS OF NATURALLY ACQUIRED PROTOZOA AND STRONGYLID NEMATODE INFECTIONS ON GROWTH AND FAECAL ATTRIBUTES IN LAMBS


8.1 INTRODUCTION

Strongylid nematodes have important impacts on sheep health, welfare and productivity worldwide (Fox, 1997; Sackett et al., 2006; Broughan and Wall, 2007; Sutherland et al., 2010) and have been associated with reduced live weight (Datta et al., 1999), growth rate (Datta et al., 1999; Macchi et al., 2001; Louie et al., 2007), diarrhoea (Broughan and Wall, 2007) and mortalities (Dargie and Allonby, 1975) in lambs. Diarrhoea is one of the most common clinical signs associated with strongylid infections in lambs (Besier and Love, 2003), but can also be caused by other pathogens including coccidia (*Eimeria*), protozoa (*Cryptosporidium* and *Giardia*), bacteria (*Campylobacter* spp., *Yersinia* spp. and *Salmonella* spp.) and viruses (Skirrow, 1994; Belloy et al., 2009). Diarrhoea results in an increased risk of breech faecal soiling of the fleece (Broughan and Wall, 2007). Breech fleece faecal soiling increases the risk of cutaneous myiasis (blowfly strike), and management of this disease increases costs and reduces productivity for sheep enterprises (Morley et al., 1976; Hall and Wall, 1995; Sackett et al., 2006). Breech fleece...
faecal soiling also increases the risk of carcase contamination, as faecal pathogens are associated with food poisoning, meat spoilage, reduced product shelf life and reduced efficiency of carcase processing (Greer et al., 1983; Hadley et al., 1997).

The intestinal protozoan parasites Cryptosporidium and Giardia are capable of infecting both domestic livestock and humans worldwide (Thompson et al., 2008; Xiao and Fayer, 2008). Protozoan infections have been commonly reported in young, naive lambs worldwide, including Australia (Yang et al., 2009). Both organisms have been associated with diarrhoea (Aloisio et al., 2006), reduced growth rate and feed intake (Olson et al., 1995; Ralston et al., 2003), depression and dehydration in lambs (Aloisio et al., 2006). However, the consequences of mixed parasite infections (strongylid nematodes and protozoan) in lambs raised specifically for meat production have not been well described.

8.1.1 AIMS AND HYPOTHESES

The aims of this study were to:

1. Investigate associations between intestinal parasites (specifically strongylid nematodes, Cryptosporidium and Giardia) with live weight, growth rate, BCS and faecal attributes of lambs grazing pastures in southern WA.

2. Utilise molecular instruments to characterise the Cryptosporidium and Giardia species/genotypes in both lamb flocks, to determine whether either flock was a potential zoonotic source for these protozoa.

3. Use molecular diagnostic techniques to differentiate the species of strongylid worm infections.

The hypotheses of this experiment were that:
1. Lambs positive for *Cryptosporidium* and/or *Giardia* will have significantly reduced live weight, growth rate and BCS, when compared to negative lambs.

2. Lambs positive for *Cryptosporidium* and/or *Giardia* will have an increased risk of producing more loose, non-pelleted faeces, when compared to negative lambs.

3. Lambs positive for a high number of internal parasite species identified (strongylids and protozoa), will have greater losses in production when compared to lambs with low numbers of internal parasite species.

4. Lambs positive for a high number of internal parasite species identified (strongylids and protozoa), will have more loose, non-pelleted faeces, when compared to lambs with low numbers of internal parasite species.

5. Adjusted WEC is negatively correlated with live weight, growth rate and BCS.

6. Adjusted WEC is positively correlated with FCS and negatively correlated with FDM%.

**8.2 MATERIALS AND METHODS**

**8.2.1 STUDY SITES, ANIMALS AND PRODUCTION MEASUREMENTS**

This experiment was approved by the Murdoch University Animal Ethics Committee (permit R2369/10). Lambs were located on two farms, Boyup Brook and Kojonup (Table 3.1, Chapter Three: Materials and Methods) in southern Western Australia, specifically in a region which experiences a Mediterranean environment characterised by hot, dry summers and cool, wet winters (Moeller *et al.*, 2008). Lamb flocks were each raised on a single paddock, the major pasture plant species being annual rye-grasses (*Lolium* spp.) and subterraneum clover (*Trifolium subterraneum*).
Lambs from each flock were randomly selected and identified with ear tags. Faecal samples were collected rectally from only these identified lambs in September (2–3 months old) and December 2010 (4–5 months old). Faecal samples were placed in individually labelled, airtight 70mL containers and stored at 2–4°C. Faecal consistency score was recorded using a scale of 1 (hard dry faecal pellet) to 5 (liquid/fluid diarrhoea) previously described (Greeff and Karlsson, 1997). Faecal dry matter percentage was measured according to the method described by the Association of Official Analytical Chemists (AOAC, 1997).

Only those lambs tagged and identified for this study had their production attributes recorded. Live weights of identified lambs from both flocks were recorded at both the first and second sampling occasions. Body condition score was recorded at only the second sampling by using a scale that ranged from 1 (very poor condition, emaciated) to 5 (excessively fat) (Sutherland et al., 2010).

8.2.2 ANTHELMINTIC TREATMENT

Lambs on both farms were treated with 12.5mg of moxidectin, 3mg selenium and 5mg vitamin B12 (Cydectin Weanerguard with Selenium and Vitamin B12, Virbac Australia) immediately after the first sampling. The second sampling occasion occurred 35 days after treatment for the Boyup Brook flock and 29 days after treatment for the Kojonup flock.

8.2.3 FAECAL WORM EGG COUNTS

Faecal worm egg counts were performed within 2 days of faecal collection using a modified McMaster WEC flotation technique with sodium chloride (specific gravity 1.20 – 1.25) as reported in the Australian Standard Diagnostic Techniques for Animal Diseases Manual (Whitlock, 1948; Lyndal-Murphy, 1993). Two grams of faeces were used from each
lamb faecal sample, with each strongylid nematode worm egg counted in a Whitlock Paracytometer Slide chamber representing 50epg. Lambs were classified as McMaster WEC positive if WEC≥50 epg.

8.2.4 DNA EXTRACTION

Genomic DNA was extracted directly from each faecal sample within seven days of collection. Genomic DNA was extracted from a sub-sample taken from the centre of each collected faecal sample. The sub-sample was weighed (250–300mg) and had genomic DNA extracted by using a Power Soil DNA Isolation Kit (MO BIO Laboratories, Inc.; 2746 Loker Avenue West; Carlsbad, CA 92010). Minor modifications to the manufacturer’s protocol were made and have been detailed in a previous study (Chapter Seven). After elution, DNA was stored at −20°C until use. Negative controls (no faecal sample), protozoa positive controls (faecal samples spiked with C. parvum and Giardia duodenalis (oo)cysts) and strongylid nematode positive controls (faecal samples with a McMaster WEC≤50 epg and spiked with a 200µL suspension containing T. circumcincta, Trichostrongylus spp., H. contortus, Oesophagostomum spp. and C. ovina L₃) were used in each faecal extraction group subset, to detect any possible contamination or any PCR inhibition. Purified DNA from both field and control samples was stored at −20°C. The transport and storage practices utilised in this study were consistent with similar studies that used PCR to detect protozoa parasites (Yang et al., 2009; Robertson et al., 2010).

8.2.5 PCR AMPLIFICATION

All samples were screened at the 18S rRNA locus for Cryptosporidium and positives were genotyped by sequencing. A two-step nested PCR protocol was used to amplify the 18S rRNA locus of Cryptosporidium previously described by Ryan et al. (2003), producing
a product of ~540bp. All Cryptosporidium positive samples at the 18S rRNA locus were confirmed by another two-step nested PCR protocol, conducted to amplify a product of ~830bp at the actin gene of Cryptosporidium, as described by Ng et al., (2006). This verified as to whether mixed infections existed (i.e. one species amplified at the 18S locus and a different species identified at the actin locus). To confirm samples detected were Cryptosporidium parvum positive, a two-step nested PCR was used to sub-genotype C. parvum positives at the 60kDa glycoprotein (gp60) gene, which amplified a fragment of ~832bp (Strong et al., 2000; Sulaiman et al., 2005).

All samples were screened for Giardia at the gdh (glutamate dehydrogenase) gene as previously described by Read et al., (2004), producing a product of ~480bp. All samples identified as positive for Giardia at the gdh gene, were also screened at the β-giardin gene with a two-step nested PCR protocol. The primary PCR reaction was performed as described by Cacciò et al., (2002), producing a ~735bp product and the secondary PCR reaction amplified a ~511bp product using primers and PCR conditions previously described Lalle et al., (2005). Positive samples were screened with nested PCRs at each of these two loci (gdh and β-giardin), to confirm the assemblage(s) detected and to determine if there were mixed G. duodenalis assemblage infections present (i.e. samples positive for different assemblages at the two different loci).

A single-step, conventional PCR assay was performed for each strongylid: T. circumcincta, Trichostrongylus spp., H. contortus and C. ovina, as described in a previous study (Bott et al., 2009). Individual forward species-specific primers (TEL, TRI, HAE and CHO) designed for the second internal transcribed spacer (ITS-2) of ribosomal DNA and the reverse primer (NC2) located at the 5’-region of the 28S rRNA gene, were used to
detect the strongylids listed above and are described in greater detail by Bott et al., (2009). The primer pair TRI-NC2, was capable of detecting all four major *Trichostrongylus* species, including *Trichostrongylus colubriformis*, *T. axei*, *T. vitrinus* and *T. rugatus* and was also capable of detecting *Oesophagostomum columbianum* and *Oesophagostomum venulosum* (Bott et al., 2009). The PCR thermocycling conditions used are described by Bott et al., (2009), with PCR reaction mixtures described in an earlier study (Chapters Five and Six). For all PCRs described above, both negative and positive controls were included.

### 8.2.6 SEQUENCE AND PHYLOGENETIC ANALYSES

Positive *Cryptosporidium* (18S rRNA, actin and *gp60*) and *Giardia* (*gdh* and β-giardin) PCR products isolated were purified using an UltraClean™ DNA Purification Kit (MO BIO Laboratories, Inc.; 2746 Loker Avenue West; Carlsbad, CA 92010) and sequenced using an ABI Prism™ Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) on an Applied Biosystems 3730 DNA Analyzer. Sequence searches were conducted using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and nucleotide sequences were analysed using Chromas Lite version 2.0 (http://www.technelysium.com.au) and alignment confirmed with reference to strongylid species from GenBank using Clustal W (http://www.clustalw.genome.jp).

Phylogenetic trees were constructed for *Cryptosporidium* isolates at 18S rRNA and actin loci and also for Giardia isolates at the *gdh* and β-giardin loci, with additional isolates obtained from GenBank. Distance estimation was performed, based on evolutionary distance calculations with the Kimura 2-parameter model and grouped firstly using TREECON software (Van de Peer and De Wachter, 1994) to conduct Neighbour-Joining analysis and secondly using Mega 5 software (Kumar et al., 2008) to conduct maximum-
parsimony analysis. The confidence of groupings from both analyses was assessed by bootstrapping, using 1000 replicates. A percentage bootstrap support of >50% was used for each phylogenetic tree constructed (Figures 8.1 and 8.2).

8.2.6 STATISTICAL ANALYSES

Statistical analysis was performed using SPSS Statistics 17.0 ( Statistical Package for the Social Sciences) for Windows (SPSS inc. Chicago, USA). Prevalences (including 95% confidence intervals) at each sampling occasion and overall prevalences (lambs positive for the respective parasite on either the first or second sampling occasion) were calculated using the exact binomial method (Thrusfield, 2007). Prevalences were compared using Pearson’s chi squared or Fisher’s exact two-sided test for independence.

McMaster WEC data were adjusted for a consistency of normally formed faeces (FCS=1) according to the following Equation 3.4 (Chapter Three: Materials and Methods) (Le Jambre et al., 2007). Adjusted WEC data were transformed using log10(adjusted WEC+25) to stabilise variances prior to statistical analysis (Dobson et al., 2009). Correlation between transformed adjusted WEC with growth rate, live weight, BCS, FCS or FDM% were analysed using a linear regression with a Pearson correlation test for significance.

Lambs were classified as uninfected (never testing positive for the parasite at either sampling) or infected (testing positive at least once for the parasite at any of the two samplings) for each parasite (Cryptosporidium, Giardia, T. circumcincta, Trichostrongylus spp., H. contortus, Oesophagostomum spp. and C. ovina) for the analysis of growth rate. Lambs were classified as positive or negative for the respective parasites at each sampling occasion for live weight, BCS, FCS and FDM% analyses. Lambs were also classified as
having single or mixed strongylid infections (lambs positive for two or more strongylid species), single or mixed Cryptosporidium and Giardia infections (lambs positive for both protozoa), protozoa negative or positive (lambs positive for either Cryptosporidium or Giardia) and single or mixed protozoan and strongylid infection (lambs positive for at least one protozoan and one strongylid species) for analyses of growth rate, live weight, BCS, FCS and FDM%.

Univariate general linear modelling (ANOVA) and least significant difference post-hoc tests were conducted for live weight, growth rate (g/day gained and % of original live weight/day gained between first and second sampling), BCS and FCS (dependent variables) with respective parasites (positive/negative, number of genera and single/mixed infection status) included as fixed factors (independent variable) and mean transformed adjusted WEC (average for the first and second sampling) included as a covariate. General linear model regression analyses and least significant difference post-hoc tests for dependent variables (growth rate, live weight, BCS, FCS and FDM%) were used with backward elimination for interactions until only significant independent variables (parasite) remained.

Odds ratio analyses and either Pearson’s chi squared test or Fisher’s exact two-sided test for independence analyses were utilised to determine association between parasites and non-pelleted faeces (FCS≥3).
8.3 RESULTS

8.3.1 INTERNAL PARASITE EPIDEMIOLOGY AND SPECIES PREVALENCE

The overall and individual sampling prevalences of all parasites are shown in Table 8.1. *Teladorsagia circumcincta* and *Trichostrongylus* spp. had the highest prevalences in both flocks. Prevalence of mixed strongylid infections was higher in the Boyup Brook than Kojonup flock on the first (P=0.004) and second (P=0.022) sampling occasions (Table 8.1). There were no significant differences in protozoan prevalences within or between farms between each sampling occasion. *Cryptosporidium* and *Giardia* species/genotypes identified are displayed in Table 8.2. *Cryptosporidium ubiquitum* and *C. parvum* were the most common species isolated from the Boyup Book and Kojonup flocks respectively (Figure 8.1). All *C. parvum* isolates from both flocks were genotyped at the *gp60* locus and found to be of the genotype IIdA20G1. Assemblage E was the most common *Giardia* genotype isolated from both flocks. All assemblage A isolates were identified as belonging to the A1 sub-assemblage group (Figure 8.2). Mixed protozoan and strongylid infection prevalence was higher at Boyup Brook on the first sampling only (P=0.033).
Table 8.1: Internal parasite prevalence (95% confidence interval) and average faecal worm egg count (WECs) for each lamb flock.

<table>
<thead>
<tr>
<th>Farm</th>
<th>Boyup Brook (n=128)</th>
<th>Kojonup (n=72)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep age</td>
<td>2-3 months</td>
<td>4-5 months</td>
</tr>
<tr>
<td>Day of study</td>
<td>0</td>
<td>35</td>
</tr>
<tr>
<td>Cryptosporidium prevalence</td>
<td>33.6 (25.5, 42.5)</td>
<td>28.1 (14.4, 35.1)</td>
</tr>
<tr>
<td>Giardia prevalence</td>
<td>30.5 (22.6, 39.2)</td>
<td>30.5 (22.6, 39.2)</td>
</tr>
<tr>
<td>Cryptosporidium and Giardia prevalence</td>
<td>17.2 (11.1, 24.9)</td>
<td>12.5 (7.3, 19.5)</td>
</tr>
<tr>
<td>H. contortus prevalence</td>
<td>7.0 (3.3, 12.9)</td>
<td>6.3 (2.7, 11.9)</td>
</tr>
<tr>
<td>T. circumcincta prevalence</td>
<td>49.2 (40.3, 58.2)</td>
<td>39.0 (30.6, 48.1)</td>
</tr>
<tr>
<td>Trichostrongylus spp. prevalence</td>
<td>34.4 (26.2, 43.3)</td>
<td>26.6 (19.1, 35.1)</td>
</tr>
<tr>
<td>C. ovina prevalence</td>
<td>10.9 (6.1, 17.7)</td>
<td>7.8 (3.8, 13.9)</td>
</tr>
<tr>
<td>Oesophagostomum spp. prevalence</td>
<td>10.2 (5.5, 16.7)</td>
<td>3.1 (0.9, 7.8)</td>
</tr>
<tr>
<td>Mixed strongylid infection prevalence</td>
<td>34.4 (26.2, 43.3)</td>
<td>28.1 (20.5, 36.8)</td>
</tr>
<tr>
<td>Mixed protozoan and nematode infection prevalence</td>
<td>32.0 (24.1, 40.9)</td>
<td>18.0 (11.7, 25.7)</td>
</tr>
<tr>
<td>Mean WEC ± S.E.M. (epg)</td>
<td>100 ± 13</td>
<td>77 ± 12</td>
</tr>
<tr>
<td>Adjusted mean WEC ± S.E.M. (epg)</td>
<td>147 ± 18</td>
<td>103 ± 15</td>
</tr>
<tr>
<td>WEC range (epg)</td>
<td>0 – 750</td>
<td>0 – 700</td>
</tr>
<tr>
<td>Adjusted mean WEC range (epg)</td>
<td>0 – 1162</td>
<td>0 – 859</td>
</tr>
</tbody>
</table>

Note: All lambs on both farms received an anthelmintic treatment following the first sampling at day 0. Mixed strongylid infections were lambs positive for two or more strongylid species. Mixed protozoa and strongylid infections were lambs positive for at least one protozoan and one strongylid species.

Adjusted mean WEC accounts for faecal consistency score (FCS).
**Table 8.2:** Cryptosporidium and Giardia species/genotypes isolated from two lamb flocks in Western Australia.

<table>
<thead>
<tr>
<th>Farm</th>
<th>Boyup Brook (n=128)</th>
<th>Kojonup (n=72)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2-3 months</td>
<td>4-5 months</td>
<td>2-3 months</td>
<td>4-5 months</td>
</tr>
<tr>
<td>Sheep age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day of study</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryptosporidium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>parvum</td>
<td>2</td>
<td>2</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td>ubiquitum</td>
<td>23</td>
<td>19</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>xiao</td>
<td>18</td>
<td>15</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>xiao and parvum mixed infection</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>43</td>
<td>36</td>
<td>23</td>
<td>17</td>
</tr>
<tr>
<td>Giardia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>assemblage E</td>
<td>17</td>
<td>21</td>
<td>14</td>
<td>11</td>
</tr>
<tr>
<td>assemblage A</td>
<td>18</td>
<td>18</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>assemblage A and E mixed infection</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>39</td>
<td>39</td>
<td>19</td>
<td>15</td>
</tr>
</tbody>
</table>
Figure 8.1: Phylogenetic relationships of Cryptosporidium species and genotypes isolated from lambs in southern Western Australia, with some known Cryptosporidium species and genotypes, as inferred by Neighbour-joining analysis of Kimura’s distances calculated from pair-wise comparisons of partial (~540bp) 18S rRNA gene (A) and (~830bp) actin gene (B) sequences. Percentage bootstrap values (>50%) from 1000 pseudoreplicate are shown for both the Neighbour-joining (first value) and maximum likelihood (second value) analyses.
Figure 8.2: Phylogenetic relationships of *Giardia duodenalis* assemblages isolated from lambs in southern Western Australia, with some known assemblages, as inferred by Neighbour-joining analysis of Kimura’s distances calculated from pair-wise comparisons of partial (~480bp) glutamate dehydrogenase (A) and (~511bp) β-Giardin (B). Percentage bootstrap values (>50%) from 1000 pseudoreplicate are shown for both the Neighbour-joining (first value) and maximum likelihood (second value) analyses. ns = node with bootstrap value <50%.
The numbers of internal parasite genera detected per lamb on each sampling occasion are shown in Figure 3. The mean number of parasite genera detected was higher at Boyup Brook than Kojonup on both the first (1.76 ± 0.11 versus 1.41 ± 0.10, P=0.001) and second (1.18 ± 0.14 versus 0.93 ± 0.13, P=0.005) sampling.

![Bar chart showing the frequency of lambs with respective total numbers of internal parasite genera detected per lamb for the Boyup Brook and Kojonup flocks.](image)

**Figure 8.3:** The frequency of lambs with respective total numbers of internal parasite genera (*Cryptosporidium*, *Giardia* and strongylid nematodes) detected per lamb for the Boyup Brook (left) and Kojonup (right) flocks.

### 8.3.2 GROWTH RATE

For both flocks, adjusted overall mean WEC was not significantly correlated with growth rate using linear regression (P>0.05). Lambs that were negative for *Trichostrongylus* spp. on both sampling occasions (n=62) had a higher growth rate (42g/day gained for a 40kg lamb) compared to *Trichostrongylus* spp.-positive lambs in the Boyup Brook flock only (P=0.003). Lambs that were negative for *Giardia* on both sampling occasions (n=53) had a higher growth rate (51g/day gain for a 40kg lamb) compared to those lambs that were *Giardia*-positive at the Kojonup flock only (P=0.033).
Significant interactions between parasites and growth rate were identified for the Kojonup flock only (Table 8.3). Mixed infections with *C. ovina* and *T. circumcincta* (n=6) or *C. ovina* and *Trichostrongylus* spp. (n=4) were both associated with weight loss. Lambs never positive for either *T. circumcincta* or *Trichostrongylus* spp. (n=36, 0.466%/day ± 0.065) had greater growth rates compared to lambs *T. circumcincta*-positive only (n=19, 0.101%/day ± 0.097), *Trichostrongylus* spp.-positive only (n=3, 0.140%/day ± 0.132) or positive for both *T. circumcincta* and *Trichostrongylus* spp. (n=14, 0.136%/day ± 0.128) (P<0.001).

**Table 8.3:** Relationships between parasites and growth rate with univariable analyses and general linear regression model (GLRM) analysis.

<table>
<thead>
<tr>
<th>Farming Property</th>
<th>Lamb growth rate (% original live weight/day gained ± SEM)</th>
<th>GLRM single and multiple parasite interactions (P value)</th>
<th>GLM $r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adjusted WEC (covariate)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Boyup Brook</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>- 0.128 ± 0.044 0.131 ± 0.046 0.106±0.050 0.162 ± 0.045 0.132±0.030 0.066 ± 0.040 0.127 ± 0.035</td>
<td>Trich (0.002)</td>
<td>0.206</td>
</tr>
<tr>
<td>Infected</td>
<td>- 0.091 ± 0.048 0.088 ± 0.046 0.113±0.043 0.050 ± 0.047 0.087±0.068 0.153 ± 0.056 0.092 ± 0.061</td>
<td>0.291</td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>0.433 0.296 0.208 0.872 0.003 0.456 0.062 0.504</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kojonup</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>- 0.445 ± 0.058 0.499 ± 0.062 0.483±0.064 0.408 ± 0.064 0.435±0.057 0.415 ± 0.047 0.527 ± 0.045</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td>- 0.425 ± 0.066 0.371 ± 0.066 0.387±0.071 0.463 ± 0.076 - 0.455 ± 0.082 0.343 ± 0.097</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>0.839 0.692 0.033 0.184 0.500 - 0.570 0.066</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: *Cryptosporidium* spp. = *Crypto*, *T. circumcincta* = *Tel*, *Trichostrongylus* spp. = *Trich*, *H. contortus* = *Haem*, *C. ovina* = *Chab*, *Oesophagostomum* spp. = *Oesoph* and SEM = standard error of the mean.
8.3.3 LIVE WEIGHT

Adjusted mean WEC was not significantly correlated with lamb live weight on either sampling occasion for both flocks. *Teladorsagia circumcincta*-negative lambs (n=65, 40.17 ± 1.16kg) were heavier than *T. circumcincta*-positive lambs (n=63, 37.06 ± 1.34kg) on the first sampling for Boyup Brook (P=0.003). In addition, *Trichostrongylus* spp.-negative lambs (n=84, 39.94 ± 1.10kg) had heavier live weights than *Trichostrongylus* spp.-positive lambs (n=44, 37.29 ± 1.40kg) (P=0.004). There were no further main effect associations between single parasite genera and live weight for either flock (Table 8.4). Boyup Brook lambs negative for both *T. circumcincta* and *Trichostrongylus* spp. (n=49) were heavier (41.42 kg ± 1.04) than *T. circumcincta* and *Trichostrongylus* spp.-positive lambs (n=28, 36.29 ± 1.36kg) and *T. circumcincta* only positive lambs (n=35, 39.93 ± 1.16kg) (P=0.038), but not *Trichostrongylus* spp. only positive lambs (n=16, 41.04 ± 1.33kg) on the first sampling occasion. No interactions between parasites were associated with live weight in the Kojonup flock.

There were no associations between mixed parasite infections (protozoa, strongylid or both) or the total number of parasite genera detected per lamb with live weight at either sampling.
Table 8.4: Relationships between parasites and live weight with univariable analyses and general linear regression model (GLRM) analysis.

<table>
<thead>
<tr>
<th>Farming Property</th>
<th>Adjusted WEC (covariate)</th>
<th>Liveweight (kg) ± SEM</th>
<th>GLRM single and multiple parasite interactions (P value)</th>
<th>GLM $r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Boyup Brook</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First Sampling</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>- 39.21 ± 1.15, 38.58 ± 1.17, 40.17 ± 1.16, 39.94 ± 1.10, 39.38 ± 0.79, 39.68 ± 0.96, 39.12 ± 0.95</td>
<td>Tel (0.002), Trich (0.030), Chab (0.077), Haem (0.090), C (0.066), Tel x Trich (0.038)</td>
<td>0.168</td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td>- 38.02 ± 1.24, 38.65 ± 1.23, 37.06 ± 1.34, 37.29 ± 1.40, 37.85 ± 1.75, 37.55 ± 1.54, 38.11 ± 1.57</td>
<td>none</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>0.404, 0.100, 0.930, 0.003, 0.004, 0.299, 0.071, 0.404</td>
<td>0.986, 0.328, 0.445, 0.554, 0.234, 0.479, 0.615, 0.896</td>
<td>0.389</td>
<td></td>
</tr>
<tr>
<td>Second Sampling</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>- 40.65 ± 2.13, 40.52 ± 2.18, 39.70 ± 1.97, 41.00 ± 2.02, 40.93 ± 1.60, 39.69 ± 1.82, 40.33 ± 1.71</td>
<td>none</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td>- 39.66 ± 2.30, 39.79 ± 2.24, 40.62 ± 2.58, 39.32 ± 2.50, 39.39 ± 3.02, 40.62 ± 2.78, 39.99 ± 3.12</td>
<td>none</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>0.986, 0.328, 0.445, 0.554, 0.234, 0.479, 0.615, 0.896</td>
<td>0.389</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Kojonup</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>First Sampling</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Uninfected</td>
<td>- 38.03 ± 1.24, 36.70 ± 1.30, 37.48 ± 1.22, 37.36 ± 1.26, 37.28 ± 1.23, 36.99 ± 1.01, 38.15 ± 1.08</td>
<td>none</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td>- 36.53 ± 1.34, 37.87 ± 1.34, 37.09 ± 1.86, 37.20 ± 1.62, - 37.58 ± 1.69, 36.42 ± 1.99</td>
<td>none</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>0.211, 0.069, 0.225, 0.841, 0.916, 0.644, 0.398</td>
<td>0.389</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second Sampling</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Uninfected</td>
<td>- 43.81 ± 2.63, 43.45 ± 2.66, 43.39 ± 1.98, 42.18 ± 2.30, 43.57 ± 2.78, 43.35 ± 2.12, 43.98 ± 2.16</td>
<td>none</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td>- 43.33 ± 3.02, 43.69 ± 3.01, 43.78 ± 3.83, 44.96 ± 3.58, - 43.79 ± 3.75, 43.16 ± 3.73</td>
<td>none</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>0.393, 0.633, 0.845, 0.883, 0.232, 0.862, 0.743</td>
<td>0.389</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Note:</strong> Cryptosporidium spp. = Crypto, T. circumcincta = Tel, Trichostrongylus spp. = Trich, H. contortus = Haem, C. ovina = Chab, Oesophagostomum spp. = Oesoph and SEM = standard error of the mean.</td>
<td></td>
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</tr>
</tbody>
</table>

8.3.4 BODY CONDITION SCORE

Mean adjusted WEC at the second sampling was not correlated with BCS for either the Boyup Brook (P=0.446) or Kojonup (P=0.175) flocks. Cryptosporidium-positive lambs (n=36) had lower BCS (2.94 ± 0.20) than Cryptosporidium-negative lambs (n=92, 3.24 ± 0.18, P=0.001) and H. contortus-positive lambs (n=8) had lower BCS (2.93 ± 0.26) than H. contortus-negative lambs (n=120, 3.25 ± 0.18, P=0.065). The proportion of variability in the data, as accounted by the GLRM model analysis, was ~13% ($r^2 = 0.127$).

Lambs positive for both protozoan genera had lower BCS (3.15 ± 0.05) than lambs negative for both protozoan genera (3.42 ± 0.05) at Boyup Brook only. Lambs identified
with mixed protozoan and strongylid infections (n=23) had lower BCSs (3.10 ± 0.10) than lambs without mixed protozoan and strongylid infection (n=105, 3.33 ± 0.05) (P=0.064). No relationship between single or multiple parasite infections and BCS were identified in the Kojonup flock. An increase in the number of parasite genera detected per lamb was associated with lower BCS at both Boyup Brook (P<0.001) and Kojonup (P=0.009) (Figure 8.4).

Figure 8.4: The mean body condition score (BCS) ± SEM for lambs with different numbers of internal parasite genera (strongylid nematodes, Cryptosporidium and Giardia) detected per lamb.

CHAPTER 8: INTERNAL PARASITES ASSOCIATED WITH PRODUCTIVITY
8.3.5 FAECAL CONSISTENCY

Lambs identified with single or mixed protozoan infections had an increased risk of non-pelleted faeces (FCS≥3) (Table 8.5). *Trichostrongylus* spp.-positive lambs (n=34) were 8.33 (1.09, 46.4) times more likely to have non-pelleted faeces than *Trichostrongylus* spp.-negative lambs in the Boyup Brook flock (P=0.005) and 6.56 (1.11, 27.2) times more likely to have non-pelleted faeces than *Trichostrongylus* spp.-negative lambs in the Kojonup flock (P=0.017) at the second sampling. No other strongylid genera were associated with a significantly increased risk of non-pelleted faeces.

Table 8.5: Risk of non-pelleted faeces (faecal consistency score [FCS] ≥ 3.0) in association with protozoan parasites.

<table>
<thead>
<tr>
<th>Farming Property</th>
<th>Odds ratio for FCS ≥3.0 (95% CI)</th>
<th>Odds ratio</th>
<th>P-value</th>
<th>Odds ratio</th>
<th>P-value</th>
<th>Odds ratio</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Cryptosporidium</em></td>
<td><em>Giardia</em></td>
<td><em>Cryptosporidium and Giardia</em></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Odds ratio</td>
<td>P-value</td>
<td>Odds ratio</td>
<td>P-value</td>
<td>Odds ratio</td>
<td>P-value</td>
<td></td>
</tr>
<tr>
<td>Boyup Brook</td>
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</tr>
<tr>
<td>First Sampling</td>
<td>2.81 (1.11 – 7.07)</td>
<td>0.025</td>
<td>2.42 (1.09 – 5.88)</td>
<td>0.070</td>
<td>4.93 (1.25 – 22.29)</td>
<td>0.036*</td>
<td></td>
</tr>
<tr>
<td>Second Sampling</td>
<td>3.75 (1.67 – 8.42)</td>
<td>0.001</td>
<td>2.87 (1.23 – 4.98)</td>
<td>0.024</td>
<td>11.84 (3.15 – 44.48)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Kojonup</td>
<td></td>
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</tr>
<tr>
<td>First Sampling</td>
<td>5.51 (1.68 – 18.07)</td>
<td>0.003</td>
<td>4.09 (1.26 – 13.23)</td>
<td>0.015</td>
<td>4.64 (1.14 – 18.81)</td>
<td>0.023</td>
<td></td>
</tr>
<tr>
<td>Second Sampling</td>
<td>11.57 (1.12 – 119.92)</td>
<td>0.039*</td>
<td>14.00 (1.34 – 146.43)</td>
<td>0.027*</td>
<td>2.91 (0.85 – 31. 84)</td>
<td>0.382*</td>
<td></td>
</tr>
</tbody>
</table>

*= Fisher’s exact test.

Adjusted mean WEC was correlated with FCS at the first (P=0.039, $r^2=0.076$) and second (P=0.018, $r^2=0.081$) sampling occasions for the Kojonup flock. This correlation represented a FCS increase from 1.4–2.1 and 1.1–1.9 across the adjusted WEC range observed (0–155 epg) on the first and second sampling, respectively. Lambs from both flocks positive for *Cryptosporidium* on either sampling had higher FCS (more loose faeces) than *Cryptosporidium*-negative lambs (P<0.001) and *Giardia*-positive lambs had higher
FCS than *Giardia*-negative lambs (P<0.05). *Trichostrongylus* spp.-positive lambs had higher FCSs than negative lambs on the second sampling only for both flocks (1.70 ± 0.91 versus 1.20 ± 0.91; P=0.005). The proportion of variability in the data accounted by the GLRM model analysis ranged between ~18–30% ($r^2 = 0.18–0.30$) (Table 8.6).

A higher number of internal parasite genera were associated with higher FCS at both Boyup Brook (P=0.032) and Kojonup (P<0.001).

**Table 8.6:** Relationships between parasites and faecal consistency score (FCS) with univariable analyses and general linear regression model (GLRM) analysis.

<table>
<thead>
<tr>
<th>Farming Property</th>
<th>FCS ± SEM</th>
<th>Adjusted WEC (covariate)</th>
<th>Crypto</th>
<th>Giardia</th>
<th>Tel</th>
<th>Trich</th>
<th>Haem</th>
<th>Chab</th>
<th>Oesoph</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Boyup Brook</strong></td>
<td></td>
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<tr>
<td>First Sampling</td>
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</tr>
<tr>
<td>Uninfected</td>
<td>- 2.41 ± 0.23</td>
<td>2.47 ± 0.23</td>
<td>2.79 ± 0.27</td>
<td>2.75 ± 0.25</td>
<td>2.83 ± 0.16</td>
<td>2.75 ± 0.21</td>
<td>2.87 ± 0.20</td>
<td>0.898</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Infected</td>
<td>- 3.04 ± 0.25</td>
<td>2.88 ± 0.25</td>
<td>2.66 ± 0.22</td>
<td>2.72 ± 0.24</td>
<td>2.62 ± 0.36</td>
<td>2.70 ± 0.31</td>
<td>2.58 ± 0.32</td>
<td>0.421</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>P-value</td>
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<td>Final Sampling</td>
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<tr>
<td>Uninfected</td>
<td>- 1.86 ± 0.32</td>
<td>2.07 ± 0.32</td>
<td>2.41 ± 0.30</td>
<td>2.19 ± 0.30</td>
<td>2.38 ± 0.28</td>
<td>2.18 ± 0.30</td>
<td>2.36 ± 0.23</td>
<td>0.079</td>
<td>0.001</td>
</tr>
<tr>
<td>Infected</td>
<td>- 2.69 ± 0.34</td>
<td>2.49 ± 0.34</td>
<td>2.34 ± 0.30</td>
<td>2.57 ± 0.31</td>
<td>1.97 ± 0.46</td>
<td>2.37 ± 0.40</td>
<td>2.19 ± 0.52</td>
<td>0.036</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>P-value</td>
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<tr>
<td><strong>Kojonup</strong></td>
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<td>First Sampling</td>
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</tr>
<tr>
<td>Uninfected</td>
<td>- 1.28 ± 0.41</td>
<td>1.53 ± 0.25</td>
<td>1.68 ± 0.32</td>
<td>1.74 ± 0.31</td>
<td>- 1.87 ± 0.22</td>
<td>1.69 ± 0.22</td>
<td>0.679</td>
<td>0.001</td>
<td>0.028</td>
</tr>
<tr>
<td>Infected</td>
<td>- 2.08 ± 0.45</td>
<td>2.07 ± 0.28</td>
<td>1.94 ± 0.26</td>
<td>1.88 ± 0.34</td>
<td>- 1.75 ± 0.38</td>
<td>1.92 ± 0.49</td>
<td>0.679</td>
<td>0.001</td>
<td>0.028</td>
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<tr>
<td>P-value</td>
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<tr>
<td>Final Sampling</td>
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</tr>
<tr>
<td>Uninfected</td>
<td>- 1.09 ± 0.21</td>
<td>1.21 ± 0.20</td>
<td>1.47 ± 0.21</td>
<td>1.20 ± 0.24</td>
<td>- 1.24 ± 0.15</td>
<td>1.47 ± 0.16</td>
<td>0.679</td>
<td>0.001</td>
<td>0.028</td>
</tr>
<tr>
<td>Infected</td>
<td>- 1.76 ± 0.22</td>
<td>1.65 ± 0.23</td>
<td>1.36 ± 0.21</td>
<td>1.70 ± 0.20</td>
<td>- 1.72 ± 0.33</td>
<td>1.37 ± 0.32</td>
<td>0.679</td>
<td>0.001</td>
<td>0.028</td>
</tr>
<tr>
<td>P-value</td>
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<tr>
<td><strong>GLRM single and multiple parasite interactions (P value)</strong></td>
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<tr>
<td>Boyup Brook</td>
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<tr>
<td>First Sampling</td>
<td>C (&lt;0.001), G (0.049)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.179</td>
<td></td>
</tr>
<tr>
<td>Final Sampling</td>
<td>C (&lt;0.001), G (0.019)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>0.210</td>
<td></td>
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<tr>
<td><strong>Kojonup</strong></td>
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</tr>
<tr>
<td>First Sampling</td>
<td>C (&lt;0.001), G (0.016), Log adjusted WEC covariate (0.082)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>0.222</td>
<td></td>
</tr>
<tr>
<td>Final Sampling</td>
<td>C (&lt;0.001), G (0.010), Log adjusted WEC covariate (0.044)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.303</td>
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</tr>
</tbody>
</table>

Note: *Cryptosporidium* spp. = *Crypto*, *T. circumcincta* = *Tel*, *Trichostrongylus* spp. = *Trich*, *H. contortus* = *Haem*, *C. ovina* = *Chab*, *Oesophagostomum* spp. = *Oesoph* and SEM = standard error of the mean.
8.3.6 FAECAL DRY MATTER

Higher adjusted WEC was correlated with reduced FDM% on both the first (30–22% across an adjusted WEC range of 0–155 epg, \( P=0.003, r^2=0.125 \)) and second (34–25%, across an adjusted WEC range of 0–155 epg, \( P=0.007, r^2=0.105 \)) sampling occasions in the Kojonup flock. No significant associations between adjusted WEC and FDM% were found in the Boyup Brook flock on either the first (\( P=0.827 \)) or second (\( P=0.504 \)) sampling occasion.

_Cryptosporidium_ and _Giardia_ were associated with higher FDM% for both flocks on both sampling occasions (Table 8.7). Lambs positive for either protozoan species had lower FDM% compared to lambs negative for both protozoan species at both Boyup Brook (21.72 ± 0.91 versus 25.42 ± 0.91; \( P=0.005 \)) and Kojonup (24.14 ± 0.89, versus 28.33 ± 0.85; \( P=0.001 \)) at the first sampling and at both Boyup Brook (21.61 ± 0.86 versus 25.64 ± 0.82; \( P<0.001 \)) and Kojonup (28.29 ± 1.14 versus 31.88 ± 0.85; \( P=0.015 \)) on the second sampling (Table 8.7).
Table 8.7: Relationships between parasites and faecal dry matter percentage (FDM%) with univariable analyses and general linear regression model (GLRM) analysis.

<table>
<thead>
<tr>
<th>Farming Property</th>
<th>Adjusted WEC (covariate)</th>
<th>Crypto</th>
<th>Giardia</th>
<th>Tel</th>
<th>Trich</th>
<th>Haem</th>
<th>Chab</th>
<th>Oesoph</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Boyup Brook</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>First Sampling</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>-</td>
<td>23.88 ± 0.86</td>
<td>24.08 ± 0.80</td>
<td>23.65 ± 1.13</td>
<td>23.08 ± 0.88</td>
<td>23.38 ± 0.69</td>
<td>23.70 ± 0.71</td>
<td>23.22 ± 0.70</td>
</tr>
<tr>
<td>Infected</td>
<td>-</td>
<td>21.11 ± 1.11</td>
<td>20.90 ± 1.12</td>
<td>25.48 ± 1.15</td>
<td>24.50 ± 1.28</td>
<td>26.11 ± 2.67</td>
<td>22.48 ± 2.06</td>
<td>26.63 ± 2.20</td>
</tr>
<tr>
<td>P-value</td>
<td>0.950</td>
<td>0.047</td>
<td>0.027</td>
<td>0.116</td>
<td>0.395</td>
<td>0.326</td>
<td>0.579</td>
<td>0.148</td>
</tr>
<tr>
<td>Final Sampling</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>-</td>
<td>24.57 ± 0.75</td>
<td>23.39 ± 0.77</td>
<td>22.90 ± 1.01</td>
<td>24.11 ± 0.80</td>
<td>23.55 ± 0.64</td>
<td>23.70 ± 0.65</td>
<td>23.72 ± 0.63</td>
</tr>
<tr>
<td>Infected</td>
<td>-</td>
<td>20.04 ± 1.12</td>
<td>21.23 ± 1.08</td>
<td>24.99 ± 1.38</td>
<td>22.64 ± 1.55</td>
<td>26.29 ± 2.69</td>
<td>23.89 ± 2.29</td>
<td>23.55 ± 3.55</td>
</tr>
<tr>
<td>P-value</td>
<td>0.729</td>
<td>0.001</td>
<td>0.095</td>
<td>0.306</td>
<td>0.445</td>
<td>0.327</td>
<td>0.936</td>
<td>0.962</td>
</tr>
<tr>
<td><strong>Kojonup</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>First Sampling</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>-</td>
<td>26.99 ± 0.83</td>
<td>26.36 ± 0.76</td>
<td>25.50 ± 1.25</td>
<td>26.25 ± 0.72</td>
<td>26.06 ± 0.72</td>
<td>26.41 ± 0.69</td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td>-</td>
<td>22.90 ± 1.09</td>
<td>23.53 ± 0.76</td>
<td>28.16 ± 2.35</td>
<td>27.16 ± 2.35</td>
<td>28.71 ± 2.06</td>
<td>25.38 ± 3.07</td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>0.005</td>
<td>0.003</td>
<td>0.048</td>
<td>0.425</td>
<td>0.721</td>
<td>-</td>
<td>0.230</td>
<td>0.745</td>
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<tr>
<td>Final Sampling</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>-</td>
<td>31.74 ± 0.76</td>
<td>31.15 ± 0.79</td>
<td>31.21 ± 0.92</td>
<td>30.97 ± 0.81</td>
<td>30.28 ± 0.70</td>
<td>30.61 ± 0.72</td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td>-</td>
<td>26.86 ± 1.40</td>
<td>28.45 ± 1.59</td>
<td>33.70 ± 1.97</td>
<td>28.46 ± 2.35</td>
<td>32.50 ± 3.48</td>
<td>30.08 ± 3.76</td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>0.013</td>
<td>0.004</td>
<td>0.141</td>
<td>0.249</td>
<td>0.349</td>
<td>-</td>
<td>0.862</td>
<td>0.891</td>
</tr>
</tbody>
</table>

GLRM single and multiple parasite interactions (P value)  

<table>
<thead>
<tr>
<th>Farming Property</th>
<th>GLM r^2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Boyup Brook</strong></td>
<td></td>
</tr>
<tr>
<td>First Sampling</td>
<td>C (0.015), G (0.006)</td>
</tr>
<tr>
<td>Final Sampling</td>
<td>C (&lt;0.001), G (0.049)</td>
</tr>
<tr>
<td><strong>Kojonup</strong></td>
<td></td>
</tr>
<tr>
<td>First Sampling</td>
<td>C (&lt;0.001), G (0.026),</td>
</tr>
<tr>
<td>Final Sampling</td>
<td>C (&lt;0.001)</td>
</tr>
</tbody>
</table>

Note: Cryptosporidium spp. = Crypto, T. circumcincta = Tel, Trichostrongylus spp. = Trich, H. contortus = Haem, C. ovina = Chab, Oesophagostomum spp. = Oesoph and SEM = standard error of the mean.
8.4 DISCUSSION

The major findings in this study were that Cryptosporidium, Giardia and Trichostrongylus spp. were associated with higher FCSs (more loose faeces) and an increased risk of lambs producing non-pelleted faeces. An increased number of parasite genera detected per lamb was associated with a lower BCS. Cryptosporidium ubiquitum and C. parvum were the Cryptosporidium species most commonly isolated from lambs, while Giardia assemblages A and E were the most common Giardia assemblages isolated. Despite high prevalences and the detection of a variety of different parasites (protozoa and strongylid nematodes) in lambs, there was little or no evidence of overt disease and so it is likely that the magnitude of these infections (number of established parasites) were below the level that would result in poor growth rates in each of the two flocks in this study.

Further studies of a larger scale are necessary to provide more information regarding the observed associations and impacts between molecular internal parasite identification and lamb productivity. While acknowledging that internal parasitism has significant consequences upon livestock production profits (Sackett et al., 2006), it is important to emphasise that other factors (such as genetics and nutrition) will have a major impact on lamb productivity and resilience to parasite challenge (Abbott et al., 1986; Kahn et al., 2003; Liu et al., 2005; Louvandini et al., 2006; Houdijk, 2008). In this present study, internal parasitism accounted for ~12–30% of the variation observed within the analyses of each flock data set ($r^2$).

Mixed strongylid infections have been reported to have greater consequences on lamb productivity than single infections (Bown et al., 1991b; Sykes and Greer, 2003). For both flocks in this present study, mixed protozoan and mixed strongylid and protozoan
infections were both associated with greater negative consequences for body condition (lower BCS) and faecal consistency (higher FCS and lower FDM% indicating more loose, wet faeces) compared to lambs with either a single infection or no evidence of internal parasite infection. The relationship between parasite infections and live weight or growth rate were complex, with a number of interactions between internal parasites identified. Lambs in this study were not subjected to fasting (held off feed) before weighing and differences in the weight of gastrointestinal contents could have impacted on the variability of live weights that were observed (Arnold and Meyer, 1988).

Assessment of BCS does not require sophisticated equipment and has been shown to be an effective measure of a sheep’s “nutritional wellbeing” and body reserves across a range of genotypes and environments (van Burgel et al., In Press). Body condition score is considered a more accurate measure of body reserves than live weight because unlike live weight, BCS is not confounded by factors such as gastrointestinal tract contents, sheep frame size, pregnancy and fleece weight (Russel et al., 1969; Warriss et al., 1987; Teixeira et al., 1989; Sanson et al., 1993; Oregui et al., 1997). Consequently BCS is potentially a more accurate and reliable indicator of the consequences of internal parasitism on productivity compared to live weight. Carcase weight was not measured in this present study, but previous studies suggest that impacts of parasitism on carcase weight may be greater than is indicated by live weight differences between infected and uninfected lambs (Jacobson et al., 2009c).

Diarrhoea outbreaks in grazing lambs are typically attributed to strongylid nematodes (Besier and Love, 2003; Sargison, 2004). In this present study, PCR detection of Trichostrongylus spp. was associated with an increased risk of non-pelleted faeces
(6.56–10.53 times) for both flocks, but only at the second sampling. *Trichostrongylus* spp have been linked to diarrhoea in grazing lambs over winter and spring months in southern Australia (Besier and Love, 2003; Woodgate and Besier, 2010). This is consistent with the present study, whereby *Trichostrongylus* spp. was associated with more loose, wet faeces. Adjusted WEC had little or no significant correlation with any of the production attributes recorded for both flocks in this study, although it was correlated positively with FCS and negatively with FDM% in the Kojonup flock.

An investigation into the epidemiology of *Cryptosporidium* and *Giardia* in each lamb flock, found that these protozoan parasites were commonly detected in lambs from both farms and that overall prevalences ranged between 26–41%. Protozoa prevalences were similar to those described in a longitudinal study of lambs in a similar geographical region (Chapter Seven). The most prevalent *Cryptosporidium* species isolated from lambs in this present study were *C. ubiquitum* and *C. xiaoi* from the Boyup Brook flock and *C. parvum* and *C. ubiquitum* from the Kojonup flock. These species have each been identified in sheep from previous studies (Santin *et al.*, 2007; Mueller-Doblies *et al.*, 2008; Yang *et al.*, 2009; Robertson *et al.*, 2010; Wang *et al.*, 2010b). Although *C. ubiquitum* has been isolated from humans worldwide (Ong *et al.*, 2002; Learmonth *et al.*, 2004; Chalmers *et al.*, 2009), it has not been detected in any human cryptosporidiosis cases in Western Australia to date (Ng *et al.*, 2010a; 2010b). *Giardia* assemblage E was the most prevalent genotype identified in both flocks, although assemblage A (Al sub-assemblage group) was isolated almost as frequently as assemblage E from the Boyup Brook flock. Assemblage E appears the most prevalent *Giardia* genotype isolated from sheep (Robertson *et al.*, 2010), although high numbers of assemblage A were isolated from lambs, in a recent study (Nolan *et al.*, 2010).
The strongylid species prevalences were similar to those reported in a recent epidemiological study conducted in the same region with *Teladorsagia circumcincta* and *Trichostrongylus* spp. most commonly identified (Chapter Six). *Haemonchus contortus* was identified in the Boyup Brook flock where environmental conditions were more favourable for the survival of *H. contortus* free-living stages compared to the Kojonup area which experiences hotter, drier summers (Besier and Dunsmore, 1993a, b; Dobson and Barnes, 1995).

In this study *Eimeria* spp. was not screened for by utilising either a microscopy or molecular technique. A recent longitudinal study in a similar geographical region to this present study, utilised microscopy to determine *Eimeria* prevalence and found no association between *Eimeria* detection and growth rate or carcass productivity in meat lambs (Chapter Nine). Future research plans to utilise molecular diagnostic techniques to screen for a number of pathogens; protozoa (including *Eimeria*), bacteria and viruses, in meat lamb faecal samples to investigate whether any pathogens are associated with reduced lamb productivity.

**8.5 CONCLUSION**

This study identified that detection of *Cryptosporidium, Giardia* and *Trichostrongylus* spp. by PCR had an impact upon faecal attributes, via associations with more loose faeces in grazing lambs. Mixed internal parasite infections and an increased number of parasite genera detected by PCR, were found to have a greater impact on lamb faecal consistency and body condition than single infections. Relationships between parasites and live weight or growth rate were complex and inconsistent. One lamb flock had high prevalences of *C. parvum* and consequently was a potential source of zoonotic *Cryptosporidium*, while the
other flock had high prevalences of assemblage A and hence was a potential source of zoonotic Giardia.
CHAPTER 9: CARCASE MEASURES ASSOCIATED WITH INTERNAL PARASITES

CRYPTOSPORIDIUM AND GIARDIA ASSOCIATED WITH REDUCED LAMB CARCASE PRODUCTIVITY


9.1 INTRODUCTION

Increased interest in the intestinal protozoa parasites Cryptosporidium and Giardia in ruminant livestock enterprises has mainly arisen because of the potential public health risks posed by these parasites and to a lesser extent their veterinary importance. Recent advances in molecular epidemiology studies have provided detailed insight into the presence of the different protozoa species/genotypes within lamb and sheep flocks worldwide (Ozdal et al., 2009; Yang et al., 2009; Robertson et al., 2010; Wang et al., 2010b).

Infections with Cryptosporidium and Giardia are most commonly reported in lambs less than 2 months of age (O'Handley and Olson, 2006; Giadinis et al., 2007; Santin et al., 2007; Yang et al., 2009), with some infections found in lambs which are only 14 days old (Castro-Hermida et al., 2001; Giadinis et al., 2007). Infections may persist for up to 16 weeks, with some animals failing to clear an initial infection or that were repeatedly re-infected from their environment or other animals (Taylor et al., 1993; Xiao, 1994; Olson et al., 1995). Infections with Giardia have been associated with reduced feed conversion...
efficiency and reduced carcase weight in surgically infected, barn-raised lambs (Olson et al., 1995) and infection with Cryptosporidium has been associated with reduced feed intake in cattle (Ralston et al., 2003), but there is little information on the productivity consequences for lambs with naturally acquired protozoan infections.

The most common clinical sign reported in sheep infected with Giardia and Cryptosporidium is diarrhoea (Olson et al., 1997; Causapé et al., 2002; Aloisio et al., 2006; Giadinis et al., 2007; Wilkes et al., 2009). However, there are a wide number of causes of diarrhoea in sheep. Strongylid nematodes have been identified as the major cause of diarrhoea and reduced productivity (including live weight and growth rate) in lambs grazed on pastures in extensive farming systems worldwide (Datta et al., 1999; van Wyk et al., 2006; Broughan and Wall, 2007; Sutherland et al., 2010). Other pathogens that have been linked with diarrhoea include Eimeria, viruses and bacteria including Campylobacter spp., Yersinia spp. and Salmonella spp. (Glastonbury, 1990; Wray et al., 1991; Skirrow, 1994; Belloy et al., 2009; Garcia et al., 2010).

Diarrhoea is a major risk factor associated with the accumulation of faeces on fleece surrounding the breech of lambs or sheep (French and Morgan, 1996; Broughan and Wall, 2007) and a significant problem for sheep meat industries worldwide, because of the increased risk of bacterial carcase contamination. This contamination leads to meat spoilage, reduced product shelf life, human food poisoning and reduced efficiency of carcase processing (Newton et al., 1978; Greer et al., 1983; Hadley et al., 1997). Fleece faecal soiling at the breech of sheep is also one of the most significant factors predisposing lambs and sheep to cutaneous myiasis (blowfly strike) (Morley et al., 1976; French et al.,
1994; Hall and Wall, 1995). It is also associated with compromised welfare for infected sheep and increased costs for sheep farmers (McLeod, 1995; Sackett et al., 2006).

9.1.1 AIMS AND HYPOTHESES

The aim of this study was to:

1. Investigate if Cryptosporidium, Giardia, Eimeria, adjusted WEC, Campylobacter jejuni or mixed protozoan infections are associated with reduced productivity in lambs grazing under extensive, broad-acre grazing conditions in WA.

The hypotheses of this experiment were that:

7. Lambs positive for Cryptosporidium and/or Giardia will have significantly reduced carcase attributes (hot carcase weight, dressing percentage and GR knife fat depth), when compared to negative lambs.

8. Lambs positive for Cryptosporidium and/or Giardia will have significantly reduced live weight, growth rate and BCS, when compared to negative lambs.

9. Lambs positive for Cryptosporidium and/or Giardia will have an increased risk of producing more loose, non-pelleted faeces, when compared to negative lambs.

10. Adjusted WEC is negatively correlated with carcase and production attributes.

11. Adjusted WEC is positively correlated with FCS and negatively correlated with FDM%.

12. Campylobacter jejuni will not be associated with any carcase, production or faecal attributes.
9.2 MATERIALS AND METHODS

9.2.1 STUDY SITES, ANIMALS AND PRODUCTION MEASUREMENTS

This experiment was approved by the Murdoch University Animal Ethics Committee (permit R2236/09). The two farms were located in Pingelly (Farm A) and Arthur River (Farm B) approximately 200 – 250km south-east of Perth, Western Australia in a region with a Mediterranean environment (hot, dry summers and cool, wet winters) (Hill et al., 2004; Moeller et al., 2008). Average annual rainfall for the two properties is between 450 – 500mm and winter stocking rates averaged between 10 – 12 dry sheep equivalents per hectare (DSE/ha) (McLaren, 1997). No cattle or goats were grazed on either property.

At marking (day 0 of study), 111 female lambs from Farm A and 124 female lambs from Farm B were randomly selected and identified with a numbered ear tag and a radio-frequency ear tag at marking. Faeces were collected directly from the rectum of only these identified female lambs using fresh latex gloves to prevent cross contamination between faecal samples on five occasions, between the first marking sampling (2 – 6 weeks of age) and final lairage sampling (7 – 8 months of age). Lambs were yarded for weighing, assessment of breech fleece faecal soiling and faecal sample collection on five sampling occasions (Table 9.1). Faecal samples were collected from lambs at the final lairage sampling 12 hours before lambs were slaughtered. A total 107 and 119 lambs from Farm A and B respectively, were sampled at all five sampling occasions.

All faecal samples were placed in individually labelled, airtight 70mL containers and transported to the laboratory within 6 hours of collection. Faecal samples were stored at 2 – 4°C and genomic DNA was extracted from each sample within seven days of collection. The transport and storage practices utilised in this study were consistent with other similar
studies that used PCR to detect these parasites (Yang et al., 2009; Ng et al., 2010a; 2010b; Robertson et al., 2010). Lamb live weight was recorded at all five sampling occasions. Body condition score was recorded at the third, fourth and final samplings by using a scale that ranged from 1 (very thin, emaciated) to 5 (excessively fat) (Sutherland et al., 2010).

Faecal attributes (FCS and FDM%) were measured at all sampling occasions. Faecal consistency score was measured using a scale of 1 (hard, dry pellet) to 5 (liquid/fluid diarrhoea) previously described (Greeff and Karlsson, 1997; Le Jambre et al., 2007). Faecal dry matter percentage was measured on fresh faeces that had been stored in air tight containers for approximately 24 hours using methods described by the Association of Official Analytical Chemists (AOAC, 1997). Breech fleece faecal soiling score was recorded at the second, third and fourth samplings (prior to crutching; removal of faecal soiled fleece from the breech area of lambs). Breech faecal soiling was measured using a scale of 1 (no evidence of breech fleece faecal soiling) to 5 (very severe breech fleece faecal soiling extending down the hind legs to, or below the hocks). A graphical illustration of these breech fleece faecal soiling scores is available in the Australian Wool Industry’s Visual Sheep Score booklet (Australian Wool Innovation et al., 2007).

Lambs were slaughtered at commercial abattoirs. Standard hot carcase weight (HCW) was recorded for lambs from Farm A and B. Fat depth at the GR site (110mm from the midline of the carcase along the lateral surface of the 12th rib on either side of the carcase) was measured with a GR knife using methods previously described (Hopkins et al., 2004) for lambs from Farm B only.
9.2.2 ANTHELMINTIC TREATMENT

Lambs were treated with 8mg Abamectin and 4mg selenium (Virbamec Oral Plus Selenium, Virbac Australia) before weaning, on days 39 and 73 of the study for Farm A and B respectively (Table 1).

9.2.3 DNA EXTRACTION

A total of 1,155 individual lamb rectal faecal samples were collected. Genomic DNA was extracted from 250 – 300mg of each faecal sample using a Power Soil DNA Kit (MolBio, West Carlsbad, California, USA) at the State Agricultural Biotechnology Centre (Murdoch University, Western Australia). Minor modifications to the manufacturer’s protocol were made and are detailed in the thesis Appendix (Chapter Thirteen). After elution, DNA was stored at –20°C until use. A negative control (no faecal sample) and a positive control (faecal sample spiked with Cryptosporidium and Giardia (oo)cysts) were used in each faecal extraction group.

Strongylid L₃ were collected from larval cultures of fresh sheep faeces. Three species of L₃ larvae (Trichostrongylus colubriformis, T. circumcincta and Haemonchus contortus) were indentified and separated into three 1.5ml Eppendorf tubes. DNA tissue extractions from approximately 200µL of the L₃ larvae suspension were performed using the DNeasy® Blood and Tissue Kit (Qiagen) following the manufacturer’s protocol (Purification of Total DNA from Animal Tissues, Qiagen). Purified DNA was stored at –20°C, until used as positive controls for strongylid worm PCRs. The strongylid PCRs had additional positive controls, with DNA extracted from faecal samples, in turn collected from lambs on the Murdoch University irrigated campus farm, known to be infected (identified by
larval culture differentiation) with all three strongylid nematodes (T. circumcincta, T. colubriformis and H. contortus).

Genomic DNA from Campylobacter jejuni was extracted from culture plates (supplied by Dr. Niki Buller, Animal Health Laboratories, Western Australia). The QIAamp® DNA Stool Mini Kit (Qiagen) was used following the manufacturer’s protocol and according to da Silva Quetz et al., (2010), except that the final eluate was 100µL of elution buffer for genomic DNA preparation (AE solution) rather than 200µL. Purified DNA was stored at –20°C, until used as positive controls for bacterial PCRs.

9.2.4 PCR AMPLIFICATION

All faecal samples and genomic livestock water DNA were screened at the 18S rRNA locus for Cryptosporidium and positives were genotyped by sequencing. A two-step nested PCR protocol was used to amplify the 18S rRNA locus of Cryptosporidium previously described by Ryan et al. (2003), producing a product of ~540bp. All Cryptosporidium positive samples at the 18S rRNA locus were confirmed by another two-step nested PCR protocol, conducted to amplify a product of ~830bp at the actin gene of Cryptosporidium, as described by Ng et al., (2006). This verified as to whether mixed infections existed (i.e. one species amplified at the 18S locus and a different species identified at the actin locus). All samples positive for Cryptosporidium mixed infections (C. xiaoii and C. parvum or C. ubiquitum and C. parvum) were further screened using a C. parvum specific qPCR at a unique Cryptosporidium specific protein coding locus previously described by Yang et al. (2009), to confirm if C. parvum was present. A two-step nested PCR was used to sub-genotype C. parvum positives at the 60kDa glycoprotein (gp60) gene, which amplified a fragment of ~832bp (Strong et al., 2000; Sulaiman et al., 2005).
All samples were screened for *Giardia* at the *gdh* (glutamate dehydrogenase) gene as previously described by Read *et al.*, (2004), producing a product of ~480bp. All samples which tested positive for *Giardia* at the *gdh* gene, were also screened at the triosephosphate isomerase (*tpi*) gene with a two-step nested PCR protocol. The primary PCR was performed as described by Sulaiman *et al.*, (2003). For the second round reaction, assemblage-specific primers and conditions for assemblage A (product ~332bp) and E (product ~388bp) were used as previously described (Geurden *et al.*, 2008a; 2009). Each of the positive samples at *gdh* gene were screened for both *G. duodenalis* assemblage E and assemblage A, to confirm the assemblage detected at the *gdh* gene and to determine if there were mixed *G. duodenalis* assemblage infections present (i.e. samples positive for different assemblages at the two loci).

A single-step PCR was performed for each sheep strongylid worm species (*T. circumcincta, Trichostrongylus* spp. and *Haemonchus contortus*) using species specific primers which amplify the second internal transcribed spacer (ITS-2) of ribosomal DNA as previously described Bott *et al.*, (2009). The PCR cycling used was described by Bott et al. (2009), with PCR reaction mixtures described in earlier Chapters Five and Six. Contamination controls, along with negative and positive controls were included.

A single step PCR protocol was used to amplify a ~290bp product of the 16S rRNA locus of *Campylobacter jejuni* as previously described Lubeck *et al.*, (2003). PCR reactions were carried out using 1µL of DNA in a 25µL reaction containing 1x PCR buffer, 2mM MgCl₂, 0.2mM dNTP, 0.4µM of each primer, 5µg BSA (20mg/mL) and 0.5U/µL of *tth*+ Taq polymerase (Fisher Biotech). Thermocycling conditions were the same as described by
Lubeck et al. (2003), except with the final extension step at 72°C lasting seven instead of four minutes.

**9.2.5 SEQUENCE ANALYSIS**

Positive *Cryptosporidium* (18S rRNA, actin and gp60), *Giardia* (gdh) and *C. jejuni* (16S rRNA) PCR products isolated were purified using an UltraClean™ DNA Purification Kit (MolBio, West Carlsbad, California, USA) and sequenced using an ABI Prism™ Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) on an Applied Biosystem 3730 DNA Analyzer. Sequence searches were conducted using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and nucleotide sequences were analysed using Chromas lite version 2.0 (http://www.technelysium.com.au) and aligned with reference genotypes from GenBank using Clustal W (http://www.clustalw.genome.jp).

**9.2.6 FAecal WORM EGG COUNTS**

Faecal worm egg counts were performed within 2 days of faecal collection using a modified McMaster technique (Lyndal-Murphy, 1993). Two grams of faeces were used from each sample and each egg counted represented 50 epg of faeces. Insufficient faecal material was available in some samples collected at the first sampling and as a result WEC data were missing for some animals at this time point (Table 9.1).

**9.2.7 STATISTICAL ANALYSIS**

Statistical analysis was performed using SPSS Statistics 17.0 (Statistical Package for the Social Sciences) for Windows (SPSS inc. Chicago, USA). All analyses were performed separately for each farm.
The WECs were adjusted for FCS to estimate WEC that would be expected if the samples were FCS=1 (consistency of normal faeces) according to the following equation by Le Jambre et al. (2007):

\[
\text{Adjusted WEC} = \frac{\text{Raw WEC}}{(34.21 - 5.15 \times \text{FCS})} \times 29.06
\]

Adjusted WEC data were assessed for normality of data distribution and homogeneity of variance. The WEC data were transformed using \(\text{Log}_{10}(\text{adjusted WEC}+25)\) to stabilise variances between groups prior to statistical analysis (Dobson et al., 2009).

Lambs that had WEC≥50 epg were classified as strongylid-positive. Lambs were classified as negative (never tested positive at any sampling occasion) or positive (tested positive on at least one sampling occasion) for each of the following: strongylids, Cryptosporidium, Giardia, Eimeria and C. jejuni. Positive-lambs were sub-categorised as positive once (positive on one occasion) or repeat positive (infected on more than one occasion). Lambs were classified according to the number of sampling occasions at which they tested positive for the above pathogens. Overall prevalences were calculated for lambs that were classified as positive for each protozoa genera and strongylid-positive by using the exact binomial method (Thrusfield, 2007).

Lamb growth rate was expressed between sequential sampling occasions as both grams gained/day (g gained/day) and using percentage liveweight change between sampling occasions (% gained/day). For analyses of the relationship between infection and growth rate (g gained/day and % gained/day), lambs classified as positive or negative at either sampling occasion were included in the calculation of the growth rate (i.e. positive at least once at either of the two sampling occasions or negative at both sampling occasions).
General linear model analyses were performed for carcase attributes (HCW, dressing percentage and GR fat depth), faecal parameters (FCS, FDM%, breech fleece faecal soiling score) or production parameters (live weight, growth rate and BCS) as dependent variables. Positive/negative parasite/bacteria classification, species/genotype present and the number of occasions when lambs tested positive for Cryptosporidium, Giardia and Eimeria were included as independent variables. Mean transformed adjusted WEC (across all sampling occasions for the respective farm) was included as a covariate. Differences in the mean measurements for carcase attributes were analysed only on the lambs sampled at all five sampling occasions using the least significant differences (LSD) post-hoc test.

Odds ratio risk analyses with Pearson’s chi squared test for independence or Fisher’s exact two-sided test for significance were conducted to determine if there was a significant association between Cryptosporidium, Giardia or Eimeria positive lambs together with faeces in a non-pelleted form (FCS≥3) and with moderate to severe (scores ≥3) breech fleece faecal soiling scores.

Correlation between transformed adjusted WECs and HCW, dressing percentage, live weight, growth rate, FDM% and FCS were analysed separately for each farm using linear regression with a Pearson one-tailed test for significance. Where significant correlations were identified, the difference across the WEC range observed was estimated using the linear regression function.
9.3 RESULTS

9.3.1 PREVALENCE OF PROTOZOA, STRONGYLID NEMATODES AND CAMPYLOBACTER JEJUNI

Prevalences of *Cryptosporidium*, *Giardia*, *Eimeria* and strongylid nematodes are reported in previous chapters and are summarised in Table 9.1. *Cryptosporidium* and *Giardia* species/genotypes are presented in Chapter Seven (Tables 7.4 and 7.5). Overall strongylid prevalences were 100% for both farms flocks (Table 9.1). *Cryptosporidium*, *Giardia*, *Eimeria* and patent *T. circumcincta* and *Trichostrongylus* spp. infections were identified on both Farm A and B at all five sampling occasions. Patent *H. contortus* infections were not detected on either farm. *Cryptosporidium* point prevalence ranged from 31.5 – 42.6% for Farm A and 18.5 – 42.0% for Farm B across the five sampling occasions. *Giardia* point prevalence ranged from 21.6 – 29.9% at Farm A and 20.3 – 29.4% at Farm B (Table 9.1). *Campylobacter jejuni* was detected in 0.9% (1/109, Farm A) and 0.8% (1/119, Farm B) of lamb faecal samples at slaughter, but was not detected in randomly selected faecal samples from either farm on any other sampling occasion (Table 9.1).
Table 9.1: Number of lambs positive for *Cryptosporidium*, *Giardia*, *Eimeria* and *Campylobacter jejuni* pathogens, along with average worm egg counts (WECs) on the two farms.

<table>
<thead>
<tr>
<th>Sampling occasion</th>
<th>Pre-weaning</th>
<th>Post-weaning</th>
<th>Overall prevalence % (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2-6 weeks</td>
<td>2 months</td>
<td>3-4 months</td>
</tr>
<tr>
<td>Farm A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lambs</td>
<td>111</td>
<td>109</td>
<td>108</td>
</tr>
<tr>
<td>Cryptosporidium</td>
<td>35</td>
<td>40</td>
<td>46</td>
</tr>
<tr>
<td>Giardia</td>
<td>24</td>
<td>29</td>
<td>30</td>
</tr>
<tr>
<td>Cryptosporidium and Giardia</td>
<td>10</td>
<td>14</td>
<td>17</td>
</tr>
<tr>
<td>Eimeria</td>
<td>7/47*</td>
<td>18</td>
<td>41</td>
</tr>
<tr>
<td>Campylobacter jejuni</td>
<td>0/20</td>
<td>0/20</td>
<td>0/20</td>
</tr>
<tr>
<td>Strongylid positive</td>
<td>14/47*</td>
<td>102</td>
<td>56</td>
</tr>
<tr>
<td>Average WEC ± S.E.M. (epg)</td>
<td>29 ± 10*</td>
<td>446 ± 45</td>
<td>73 ± 13</td>
</tr>
<tr>
<td>Adjusted average WEC a ±</td>
<td>32 ± 11*</td>
<td>552 ± 59</td>
<td>89 ± 18</td>
</tr>
<tr>
<td>S.E.M. (epg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Farm B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lambs</td>
<td>124</td>
<td>123</td>
<td>123</td>
</tr>
<tr>
<td>Cryptosporidium</td>
<td>23</td>
<td>36</td>
<td>48</td>
</tr>
<tr>
<td>Giardia</td>
<td>29</td>
<td>25</td>
<td>32</td>
</tr>
<tr>
<td>Cryptosporidium and Giardia</td>
<td>8</td>
<td>7</td>
<td>17</td>
</tr>
<tr>
<td>Eimeria</td>
<td>7/41*</td>
<td>24</td>
<td>62</td>
</tr>
<tr>
<td>Campylobacter jejuni</td>
<td>0/20</td>
<td>0/20</td>
<td>0/20</td>
</tr>
<tr>
<td>Strongylid positive</td>
<td>14/41*</td>
<td>74</td>
<td>102</td>
</tr>
<tr>
<td>Average WEC ± S.E.M. (epg)</td>
<td>27 ± 7*</td>
<td>87 ± 8</td>
<td>211 ± 18 9</td>
</tr>
<tr>
<td>Adjusted average WEC a ±</td>
<td>29 ± 7*</td>
<td>118 ± 12</td>
<td>273 ± 22 7</td>
</tr>
<tr>
<td>S.E.M. (epg)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*= Not all samples had enough faecal material to conduct WEC at his sampling occasion.

a = Faecal worm egg counts adjusted for faecal consistency.

† = Indicates after this sampling occasion that lambs received an anthelmintic treatment.

Lambs with a McMaster WEC≥50 epg were classified as strongylid positive.
9.3.2 CARCASE ATTRIBUTES

Detection of Cryptosporidium on at least one sampling occasion was associated with 1.25kg (6.6%) lower HCW for Farm A (P=0.029) and 1.65kg (11.0%) lower HCW for Farm B (P<0.001) (Table 9.2). Lambs positive for Cryptosporidium on two or more occasions had 1.02kg (5.4%) and 1.4kg (9.3%) lower HCW compared to lambs positive for Cryptosporidium once or never at all for Farm A (P=0.015, F=6.10) and Farm B (P<0.001, F=17.43) respectively. There was a trend to 0.69kg (4.6%) lower HCW in lambs positive for Giardia on at least one sampling occasion for Farm B (P=0.065), but not for Farm A (Table 9.2).

Detection of Cryptosporidium on at least one sampling occasion was associated with 1.7% and 1.9% lower dressing percentages for Farm A (P=0.022) and Farm B (P<0.001) respectively (Table 9.2). Lambs positive for Cryptosporidium on two or more occasions had 1.6% lower dressing percentages compared to lambs positive for Cryptosporidium once or never at all for both Farms A (P<0.001, F=8.93) and B (P<0.001, F=12.12). Detection of Giardia on at least one sampling occasion was associated with 1.7% lower dressing percentages on Farm B (P<0.001), but not on Farm A (Table 9.3). Lambs positive for both Cryptosporidium and Giardia on more than one sampling occasion had 1.9% lower dressing percentages (37.5% ± 0.6) compared to lambs never positive for both protozoa or positive for both on only one occasion (39.4% ± 0.5%) for Farm B (P<0.001, F=11.84).

Lambs positive for Giardia on four sampling occasions had lower GR fat depth (2.86 ± 0.84mm) compared to lambs in which Giardia was never detected (4.48 ± 0.34mm; P=0.049) (Table 9.4). Detection of Cryptosporidium or Giardia on at least one occasion
was not associated with different GR fat depth for Farm B. No GR fat depth measurements were recorded for lambs from Farm A.

Lambs positive for both *Cryptosporidium* and *Giardia* at least once out of the five samplings had significantly lighter HCWs by 1.6kg (10.7%) compared to lambs never positive for both *Cryptosporidium* and *Giardia* on Farm B only (P=0.001) (Tables 9.3 and 9.4). Dressing percentages were 1.9% (P<0.001) and 1.1% (P=0.056) lower in lambs positive for both *Cryptosporidium* and *Giardia* on at least one of the five samplings, compared to lambs never testing positive to both protozoa for Farm A and B respectively (Tables 9.3 and 9.4).

There were no significant associations between carcase attributes (HCW, dressing % or GR fat depth) and adjusted WEC or *Eimeria* for either Farm A or B.
Table 9.2: Associations between the detection of *Cryptosporidium* or *Giardia* at least once with hot carcase weight (HCW) and dressing percentage (%).

<table>
<thead>
<tr>
<th>Carcase attribute, parasite and farming property</th>
<th>Carcase attributes (mean ± standard error)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protozoa-negative</td>
<td>Protozoa-positive</td>
<td>P-value</td>
<td>F-value</td>
</tr>
<tr>
<td>Hot Carcase Weight (kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cryptosporidium</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Farm A</td>
<td>19.85 ± 0.49</td>
<td>18.60 ± 0.24</td>
<td>0.029</td>
<td>5.05</td>
</tr>
<tr>
<td>Farm B</td>
<td>16.14 ± 0.33</td>
<td>14.49 ± 0.21</td>
<td>&lt;0.001</td>
<td>13.85</td>
</tr>
<tr>
<td><em>Giardia</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Farm A</td>
<td>19.35 ± 0.38</td>
<td>18.58 ± 0.26</td>
<td>0.101</td>
<td>2.74</td>
</tr>
<tr>
<td>Farm B</td>
<td>15.42 ± 0.29</td>
<td>14.73 ± 0.24</td>
<td>0.063</td>
<td>3.53</td>
</tr>
<tr>
<td><em>Cryptosporidium and Giardia</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Farm A</td>
<td>19.04 ± 0.28</td>
<td>18.47 ± 0.34</td>
<td>0.201</td>
<td>1.66</td>
</tr>
<tr>
<td>Farm B</td>
<td>15.56 ± 0.21</td>
<td>13.98 ± 0.30</td>
<td>&lt;0.001</td>
<td>18.67</td>
</tr>
<tr>
<td>Dressing Percentage (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cryptosporidium</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Farm A</td>
<td>44.44 ± 0.66</td>
<td>42.72 ± 0.31</td>
<td>0.022</td>
<td>5.44</td>
</tr>
<tr>
<td>Farm B</td>
<td>39.07 ± 0.45</td>
<td>37.21 ± 0.28</td>
<td>&lt;0.001</td>
<td>12.68</td>
</tr>
<tr>
<td><em>Giardia</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Farm A</td>
<td>43.70 ± 0.50</td>
<td>42.71 ± 0.34</td>
<td>0.104</td>
<td>2.69</td>
</tr>
<tr>
<td>Farm B</td>
<td>38.74 ± 0.38</td>
<td>37.06 ± 0.31</td>
<td>&lt;0.001</td>
<td>11.86</td>
</tr>
<tr>
<td><em>Cryptosporidium and Giardia</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Farm A</td>
<td>43.52 ± 0.36</td>
<td>42.41 ± 0.44</td>
<td>0.056</td>
<td>3.73</td>
</tr>
<tr>
<td>Farm B</td>
<td>38.40 ± 0.29</td>
<td>36.53 ± 0.40</td>
<td>&lt;0.001</td>
<td>14.21</td>
</tr>
</tbody>
</table>

Note: A total of 107 and 119 lambs were sampled at all five sampling occasions for Farm A and B respectively.
**Table 9.3:** Associations between *Cryptosporidium, Giardia* or both detection frequencies in lamb faeces collected across all five sampling occasions with carcase productivity at slaughter (mean ± standard error).

<table>
<thead>
<tr>
<th>Carcase attribute, farming property and parasite</th>
<th>Frequency of sampling occasions lambs tested positive for protozoa parasites (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hot Carcase Weight (kg)</td>
<td>0                        1       2       3       4       5</td>
</tr>
<tr>
<td><strong>Cryptosporidium</strong></td>
<td></td>
</tr>
<tr>
<td>Farm A</td>
<td>19.85 ± 0.49A</td>
</tr>
<tr>
<td>Farm B</td>
<td>16.14 ± 0.33A</td>
</tr>
<tr>
<td><strong>Giardia</strong></td>
<td></td>
</tr>
<tr>
<td>Farm A</td>
<td>19.35 ± 0.38ACL</td>
</tr>
<tr>
<td>Farm B</td>
<td>15.42 ± 0.29G</td>
</tr>
<tr>
<td><strong>Cryptosporidium and Giardia</strong></td>
<td></td>
</tr>
<tr>
<td>Farm A</td>
<td>19.05 ± 0.28A</td>
</tr>
<tr>
<td>Farm B</td>
<td>15.56 ± 0.21A</td>
</tr>
<tr>
<td><strong>Dressing percentage (%)</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Cryptosporidium</strong></td>
<td></td>
</tr>
<tr>
<td>Farm A</td>
<td>44.44 ± 0.66A</td>
</tr>
<tr>
<td>Farm B</td>
<td>39.07 ± 0.45A</td>
</tr>
<tr>
<td><strong>Giardia</strong></td>
<td></td>
</tr>
<tr>
<td>Farm A</td>
<td>43.70 ± 0.50A</td>
</tr>
<tr>
<td>Farm B</td>
<td>38.74 ± 0.38A</td>
</tr>
<tr>
<td><strong>Cryptosporidium and Giardia</strong></td>
<td></td>
</tr>
<tr>
<td>Farm A</td>
<td>43.51 ± 0.37A</td>
</tr>
<tr>
<td>Farm B</td>
<td>38.40 ± 0.29AC</td>
</tr>
<tr>
<td><strong>Lambs (N)</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Cryptosporidium</strong></td>
<td></td>
</tr>
<tr>
<td>Farm A</td>
<td>20</td>
</tr>
<tr>
<td>Farm B</td>
<td>33</td>
</tr>
<tr>
<td><strong>Giardia</strong></td>
<td></td>
</tr>
<tr>
<td>Farm A</td>
<td>36</td>
</tr>
<tr>
<td>Farm B</td>
<td>45</td>
</tr>
<tr>
<td><strong>Cryptosporidium and Giardia</strong></td>
<td></td>
</tr>
<tr>
<td>Farm A</td>
<td>64</td>
</tr>
<tr>
<td>Farm B</td>
<td>78</td>
</tr>
</tbody>
</table>

*abcd* Values in rows with different superscripts are significantly different (P<0.05).

Note: A total of 107 and 119 lambs were sampled at all five sampling occasions for Farm A and B respectively.

CHAPTER 9: CARCASE MEASURES ASSOCIATED WITH INTERNAL PARASITES
Table 9.4: General linear model analysis of significant (P<0.100) interactions identified between *Cryptosporidium*, *Giardia*, and *Eimeria* for different production and faecal attributes.

<table>
<thead>
<tr>
<th>Farming property and production or faecal attributes</th>
<th>General Linear Model significance (P value)</th>
<th>WEC covariate</th>
<th>C</th>
<th>G</th>
<th>E</th>
<th>CxG</th>
<th>CxE</th>
<th>GxE</th>
<th>CxGxE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Farm A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dressing percentage</td>
<td>-</td>
<td>-</td>
<td>0.022</td>
<td>-</td>
<td>-</td>
<td>0.056</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sampling 4 Live weight (kg)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.038</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.048</td>
</tr>
<tr>
<td>Sampling 4 BCS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.011</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.003</td>
</tr>
<tr>
<td>Sampling 3 BCS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.084</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sampling 3 Breech fleece soiling score</td>
<td>&lt;0.001</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.048</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Farm B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCW</td>
<td>-</td>
<td>-</td>
<td>0.029</td>
<td>0.063</td>
<td>-</td>
<td>0.001</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dressing percentage</td>
<td>-</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Multiple infections on Dressing percentage</td>
<td>-</td>
<td>0.041</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.014</td>
<td>-</td>
<td>-</td>
<td>0.011</td>
</tr>
<tr>
<td>Multiple infections on GR FAT (mm)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.085</td>
<td></td>
</tr>
<tr>
<td>Sampling 5 Live weight (kg)</td>
<td>-</td>
<td>0.088</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.009</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sampling 3 Live weight (kg)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.011</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sampling 2 Live weight (kg)</td>
<td>-</td>
<td>0.003</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.043</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sampling 5 BCS</td>
<td>-</td>
<td>0.072</td>
<td>0.039</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.039</td>
<td></td>
</tr>
<tr>
<td>Sampling 4 Breech fleece soiling score</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.078</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sampling 3 Faecal consistency score</td>
<td>&lt;0.001</td>
<td>0.006</td>
<td>-</td>
<td>0.062</td>
<td>-</td>
<td>0.030</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

9.3.3 LIVE WEIGHT AND GROWTH RATE

Detection of *Cryptosporidium* or *Giardia* was associated with adverse consequences for live weight and growth rate, but these observations were not consistent across sampling occasions, farms and protozoa genera. In the unweaned lambs, the average liveweight change (percentage of starting live weight) between the first marking sampling and the second sampling was 0.23%/day lower in *Cryptosporidium*-positive lambs compared to *Cryptosporidium*-negative lambs for both Farm A (P=0.041) and Farm B (P=0.037). This
reflected a 23g/day difference in growth rate over this period for a lamb weighing 10kg at marking. Average growth rate (g/day) was 38g/day lower in Cryptosporidium-positive lambs compared to Cryptosporidium-negative lambs between the first marking sampling and the second sampling on Farm A (P<0.001) but not Farm B. On Farm B, detection of Cryptosporidium on the sampling occasion either immediately before (third sampling) or after weaning (fourth sampling) was associated with 0.16%/day lower growth rate compared with lambs negative for Cryptosporidium at these occasions (P=0.049). This difference reflected a 20.6g/day difference in growth rate for a 33kg lamb (weaning weight) over this period.

Lambs positive for both Cryptosporidium and Giardia (37.32 ± 1.15kg) were lighter than lambs positive for Giardia only (44.53 ± 2.19kg) on the final (slaughter) sampling for Farm B (P=0.013) (Table 9.5).

No other significant effects of protozoa on live weight and growth rate were observed (Table 9.5).

There was no significant correlation between adjusted WEC and live weight or growth rate on any of the sampling occasions for both farms. There was no difference in the live weight or growth rate of lambs that were strongylid-positive compared to strongylid-negative lambs on any sampling at either farm.
Table 9.5: Associations between detection of *Cryptosporidium* or *Giardia* in lamb faeces with live weights at each of the five sampling occasions.

<table>
<thead>
<tr>
<th>Parasite and farming property</th>
<th>Live weight (mean ± standard error)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protozoa-negative</td>
<td>Protozoa-positive</td>
<td>P-value</td>
<td>F-value</td>
<td></td>
</tr>
<tr>
<td>Cryptosporidium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Farm A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sampling 1</td>
<td>12.37 ± 0.41</td>
<td>11.09 ± 0.52</td>
<td>0.064</td>
<td>3.48</td>
<td></td>
</tr>
<tr>
<td>Sampling 2</td>
<td>21.89 ± 0.37</td>
<td>19.79 ± 0.49</td>
<td>&lt;0.001</td>
<td>11.43</td>
<td></td>
</tr>
<tr>
<td>Sampling 3</td>
<td>30.69 ± 0.50</td>
<td>30.23 ± 0.58</td>
<td>0.548</td>
<td>0.36</td>
<td></td>
</tr>
<tr>
<td>Sampling 4</td>
<td>41.54 ± 0.48</td>
<td>40.64 ± 0.62</td>
<td>0.471</td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td>Sampling 5</td>
<td>43.98 ± 0.51</td>
<td>43.15 ± 0.67</td>
<td>0.330</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td><strong>Farm B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sampling 1</td>
<td>15.87 ± 0.27</td>
<td>14.18 ± 0.57</td>
<td>0.008</td>
<td>7.23</td>
<td></td>
</tr>
<tr>
<td>Sampling 2</td>
<td>28.96 ± 0.40</td>
<td>26.28 ± 0.61</td>
<td>0.003</td>
<td>8.91</td>
<td></td>
</tr>
<tr>
<td>Sampling 3</td>
<td>33.79 ± 0.55</td>
<td>32.96 ± 0.69</td>
<td>0.350</td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td>Sampling 4</td>
<td>39.39 ± 0.49</td>
<td>37.51 ± 0.69</td>
<td>0.029</td>
<td>4.91</td>
<td></td>
</tr>
<tr>
<td>Sampling 5</td>
<td>42.07 ± 0.68</td>
<td>40.25 ± 0.76</td>
<td>0.088</td>
<td>2.95</td>
<td></td>
</tr>
</tbody>
</table>

*Giardia*

| **Farm A**                    |                        |                        |          |          |
| Sampling 1                    | 11.88 ± 0.37           | 11.90 ± 0.77           | 0.971    | 0.01     |
| Sampling 2                    | 20.96 ± 0.37           | 21.51 ± 0.62           | 0.462    | 0.54     |
| Sampling 3                    | 30.59 ± 0.45           | 30.63 ± 0.72           | 0.707    | 0.15     |
| Sampling 4                    | 41.97 ± 0.63           | 39.35 ± 0.91           | 0.036    | 4.50     |
| Sampling 5                    | 43.80 ± 0.49           | 43.40 ± 0.75           | 0.655    | 0.20     |

| **Farm B**                    |                        |                        |          |          |
| Sampling 1                    | 16.01 ± 0.41           | 17.19 ± 0.84           | 0.219    | 1.56     |
| Sampling 2                    | 27.47 ± 0.38           | 28.41 ± 0.75           | 0.376    | 0.79     |
| Sampling 3                    | 33.69 ± 0.38           | 32.81 ± 0.75           | 0.371    | 0.81     |
| Sampling 4                    | 39.45 ± 0.45           | 37.94 ± 0.71           | 0.070    | 3.29     |
| Sampling 5                    | 40.07 ± 0.46           | 38.93 ± 0.72           | 0.188    | 1.75     |

### 9.3.4 BODY CONDITION SCORE

Detection of protozoa was associated with lower BCS, but this was not consistent across sampling occasions, farms and protozoa genera. *Cryptosporidium*-positive lambs
had 0.42 lower BCS at the fourth sampling for Farm A (2.70 ± 0.08 versus 3.12 ± 0.06; 
P=0.011) and a trend towards 0.41 lower BCS at the final sampling for Farm B (2.43 ± 0.07 
versus 2.84 ± 0.06; P=0.072) compared with *Cryptosporidium*-negative lambs. *Giardia-
positive lambs had 0.26 lower BCS on the final sampling for Farm B (2.50 ± 0.09 versus 
2.76 ± 0.06; P=0.039) (Table 9.6). Lambs positive for *Cryptosporidium*, *Giardia* and 
*Eimeria* (mixed infection) had 1.09 and 0.88 lower BCS than lambs negative for all three 
protozoa at the fourth sampling for Farm A (P=0.002) and final sampling for Farm B 
(P=0.022) respectively (Table 9.6).

   No other association between BCS and detection of *Cryptosporidium*, *Giardia*, 
*Eimeria* or adjusted WEC were identified on any other sampling occasion for either farm.
Table 9.6: Associations between detection of *Cryptosporidium* or *Giardia* in lamb faeces with live weights at each of the five sampling occasions.

<table>
<thead>
<tr>
<th>Farming property and parasite</th>
<th>BCS at each sampling occasion</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protozoa-negative</td>
<td>Protozoa-positive</td>
<td>P-value</td>
<td>F-value</td>
</tr>
<tr>
<td><strong>Farm A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryptosporidium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sampling 3</td>
<td>2.67 ± 0.07</td>
<td>2.59 ± 0.08</td>
<td>0.433</td>
<td>0.62</td>
</tr>
<tr>
<td>Sampling 4</td>
<td>3.12 ± 0.06</td>
<td>2.70 ± 0.08</td>
<td>0.011</td>
<td>4.67</td>
</tr>
<tr>
<td>Sampling 5</td>
<td>3.19 ± 0.06</td>
<td>3.14 ± 0.08</td>
<td>0.595</td>
<td>0.28</td>
</tr>
<tr>
<td>Giardia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sampling 3</td>
<td>2.64 ± 0.06</td>
<td>2.61 ± 0.09</td>
<td>0.798</td>
<td>0.07</td>
</tr>
<tr>
<td>Sampling 4</td>
<td>2.96 ± 0.06</td>
<td>2.89 ± 0.08</td>
<td>0.425</td>
<td>0.64</td>
</tr>
<tr>
<td>Sampling 5</td>
<td>3.20 ± 0.05</td>
<td>3.11 ± 0.08</td>
<td>0.404</td>
<td>0.70</td>
</tr>
<tr>
<td><strong>Farm B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryptosporidium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sampling 3</td>
<td>3.15 ± 0.06</td>
<td>2.72 ± 0.08</td>
<td>0.095</td>
<td>2.57</td>
</tr>
<tr>
<td>Sampling 4</td>
<td>2.51 ± 0.06</td>
<td>2.39 ± 0.08</td>
<td>0.219</td>
<td>1.53</td>
</tr>
<tr>
<td>Sampling 5</td>
<td>2.86 ± 0.05</td>
<td>2.55 ± 0.06</td>
<td>0.093</td>
<td>2.61</td>
</tr>
<tr>
<td>Giardia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sampling 3</td>
<td>3.00 ± 0.08</td>
<td>2.82 ± 0.10</td>
<td>0.288</td>
<td>1.14</td>
</tr>
<tr>
<td>Sampling 4</td>
<td>2.50 ± 0.06</td>
<td>2.39 ± 0.09</td>
<td>0.282</td>
<td>1.17</td>
</tr>
<tr>
<td>Sampling 5</td>
<td>2.76 ± 0.06</td>
<td>2.50 ± 0.09</td>
<td>0.039</td>
<td>4.35</td>
</tr>
</tbody>
</table>

9.3.5 FAECAL CONSISTENCY AND DRY MATTER

Protozoa detection and increased WEC were associated with more loose, wetter faeces, but these findings were not consistent across parasite genera, sampling occasions or farms.

Unweaned *Cryptosporidium*-positive lambs were 4.7 (1.35 – 16.52, 95% CI) times and 3.8 (1.62 – 9.09) times more likely to have non-pelleted faeces (FCS ≥3.0) than *Cryptosporidium*-negative lambs at the second sampling for Farm A (P=0.002) and Farm B.
(P=0.006), respectively. Also, unweaned Cryptosporidium-positive lambs were 3.1 (1.36 – 7.18) times more likely to have non-pelleted faeces than Cryptosporidium-negative lambs at the third sampling for Farm B (P=0.006) (Table 9.7). There was a trend towards 0.75 higher FCS in lambs in which C. ubiquitum was isolated compared to lambs in which other Cryptosporidium species were detected (P=0.092, F=2.24). Giardia-positive lambs were 3.1 (1.26 – 6.83) times more likely to have non-pelleted faeces than lambs Giardia-negative lambs at the final sampling for Farm A (P=0.042). Lambs positive for G. duodenalis assemblage A (either as a single infection or in combination with assemblage E) had 1.0 – 1.5 higher FCS than lambs positive for Assemblage E only at the second, third and final sampling occasions for Farm B (P<0.001). Eimeria-positive lambs were 3.6 (1.48 – 8.56) times more likely to have non-pelleted faeces than Eimeria-negative lambs at the third sampling for Farm B (P=0.003). Lambs positive for both Cryptosporidium and Eimeria had higher FCS (2.78 ± 0.22) than lambs negative for both parasites (1.76 ± 0.12) at the third sampling for Farm B (P<0.001) (Table 9.7).

Adjusted WECs were positively correlated with FCS at the first (Farm A), second (Farm A and Farm B), third (Farm B), fourth (Farm A and Farm B) and final (Farm A and Farm B) sampling occasions (Table 9.7). Where significant correlations were observed, the $r^2$ ranged from 0.08 to 0.36 (Table 9.7). The largest change in FCS across the adjusted WEC range observed was a FCS increase from 1.2 – 4.0 (P<0.001, $r^2$=0.36) over the adjusted WEC range 0 – 4000 epg observed on the final sampling for Farm A.

Adjusted WEC was negatively correlated with FDM% at the second sampling for both Farm A (P=0.014, $r^2$=0.13) and Farm B (P=0.021, $r^2$=0.16), third sampling for Farm B only (P=0.024, $r^2$=0.12) and final sampling for Farm A only (P=0.026, $r^2$=0.19). The largest
change in FDM% was 18% (40 – 22%) (P=0.026, \( r^2=0.19 \)) over the adjusted WEC range 0 – 4000 epg observed on the final sampling for Farm A.

**Table 9.7:** Risk of non-pelleted faeces (faecal consistency score [FCS] ≥ 3.0) in sheep positive for protozoa parasites and correlation between adjusted worm egg count (WEC) and FCS.

<table>
<thead>
<tr>
<th>Farm A</th>
<th>Odds ratio for FCS ≥3.0 (95% CI) with respective p-value</th>
<th>Adjusted WEC and FCS regression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cryptosporidium</td>
<td>Giardia</td>
</tr>
<tr>
<td>Sampling 1</td>
<td>0.71 (0.62 – 2.15)</td>
<td>0.495*</td>
</tr>
<tr>
<td>Sampling 2</td>
<td>4.72 (1.35 – 16.52)</td>
<td>0.010</td>
</tr>
<tr>
<td>Sampling 3</td>
<td>2.18 (0.76 – 6.23)</td>
<td>0.140</td>
</tr>
<tr>
<td>Sampling 4</td>
<td>0.88 (0.38 – 2.05)</td>
<td>0.762</td>
</tr>
<tr>
<td>Sampling 5</td>
<td>1.47 (0.66 – 3.28)</td>
<td>0.340</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Farm B</th>
<th>Odds ratio for FCS ≥3.0 (95% CI) with respective p-value</th>
<th>Adjusted WEC and FCS regression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cryptosporidium</td>
<td>Giardia</td>
</tr>
<tr>
<td>Sampling 1</td>
<td>4.55 (0.27 – 75.00)</td>
<td>0.338*</td>
</tr>
<tr>
<td>Sampling 2</td>
<td>3.84 (1.62 – 9.09)</td>
<td>0.002</td>
</tr>
<tr>
<td>Sampling 3</td>
<td>3.13 (1.36 – 7.18)</td>
<td>0.006</td>
</tr>
<tr>
<td>Sampling 4</td>
<td>0.56 (0.15 – 2.16)</td>
<td>0.395</td>
</tr>
<tr>
<td>Sampling 5</td>
<td>1.49 (0.60 – 3.68)</td>
<td>0.387</td>
</tr>
</tbody>
</table>

* = Fisher’s exact test

### 9.3.6 BREECH FLEECE FAECAL SOILING

Protozoa detection and increased WEC were associated with higher breech fleece faecal soiling scores, but these findings were not consistent across sampling occasions, farms and protozoa genera.

Unweaned *Cryptosporidium*-positive lambs were 3.4 (1.41 – 6.98) times and 3.6 (1.44 – 7.27) times more likely to have moderate or severe breech fleece faecal soiling (score of 3.0 or higher) than *Cryptosporidium*-negative lambs on the second sampling
occasion for Farm A (P=0.026) and B (P=0.014), respectively. Weaned *Cryptosporidium*-positive lambs were 3.0 (1.27 – 6.07) times more likely to have moderate to severe breech fleece faecal soiling than *Cryptosporidium*-negative lambs on the fourth sampling occasion for Farm A (P=0.047). Lambs in which *C. ubiquitum* was isolated had approximately a 0.6 higher breech fleece faecal soiling score than lambs from which other *Cryptosporidium* species were isolated (P=0.043, F=3.417).

Significant positive correlations between adjusted WEC and breech fleece faecal soiling score were only identified in unweaned lambs on Farm A at the second sampling, where breech fleece faecal soiling score increased from 1.5 – 3.1 over an adjusted WEC range 0 – 3950 epg ($r^2=0.14$, P=<0.001). A similar correlation on Farm A at the third sampling was found, where breech fleece faecal soiling score increased from 1.5 – 3.1 over an adjusted WEC range 0 – 1100 epg ($r^2=0.11$, P<0.001).
9.4 DISCUSSION

This study identified reduced carcase productivity for lambs that tested positive at least once for *Cryptosporidium* or *Giardia* between marking (2 – 6 weeks old) and slaughter (7 – 8 months). Reduced HCWs and dressing percentages were associated with *Cryptosporidium* on both farms and *Giardia* on one farm. Both HCW and dressing percentage are important profit drivers for sheep meat producers and processors, therefore these findings are of importance to the sheep meat industry and also potentially other livestock industries in which *Cryptosporidium* and *Giardia* infections have been identified. Reduced live weight, growth weight, body condition, more loose faeces and greater breech fleece faecal soiling were also observed in lambs which tested positive for *Cryptosporidium* and *Giardia*, but these associations were inconsistent across sampling occasions, farms and parasites investigated in this study. However, recent studies suggest that live weight and liveweight change may underestimate the impact of parasitism on carcase productivity in sheep challenged with strongylid nematode larvae and therefore HCW may be a more appropriate measure in determining the true economic impact of parasitism in sheep meat enterprises (Liu *et al*., 2005; Jacobson *et al*., 2009c). Although parasitism may have significant effects upon production, it is important to emphasise that other important factors (including genetics and nutrition) impact strongly on lamb productivity (Abbott *et al*., 1986; Bown *et al*., 1991a; Coop and Sykes, 2002; Kahn *et al*., 2003; Liu *et al*., 2005; Louvandini *et al*., 2006; Houdijk, 2008).

This is the first study reporting associations between lamb production attributes and naturally acquired *Cryptosporidium* and *Giardia* in lambs grazing pastures in extensive broad-acre environments. Associations between these protozoa and animal performance have been studied in laboratory animals, feedlot steers and surgically infected, barn-raised
There are few reports of the consequences of protozoa infections on carcase attributes in livestock. Olson et al. (1995) observed reduced feed conversion efficiency and a 1.3kg (6.1%) reduction in carcase weight for specific pathogen free, barn-raised lambs that were surgically infected with *Giardia* trophozoites, suggesting that malabsorption and maldigestion contributed towards reduced energy available for growth of the lambs. Feed intake and feed conversion efficiency were not measured in this present study.

Protozoa have been reported to cause inflammatory changes in the gastrointestinal tract (Buret et al., 2002; Scott et al., 2002; Buret et al., 2003). Other studies have observed heavier intestinal weight (as a proportion of their live weight) in sheep challenged with strongylid nematode larvae (Liu et al., 2005; Jacobson et al., 2009c), suggesting that measurement of live weight may underestimate the carcase productivity consequences associated with parasite infections. Other factors that may impact dressing percentages include time off feed, gender, breed, time of weaning, weight of skins and wool length of lambs (Makarechian et al., 1978; Arnold and Meyer, 1988), all of which were consistent within the two flocks in this study.

*Giardia* infection has been associated with reduced feed intake in steers (Ralston et al., 2003), although a number of studies failed to identify any association between *Giardia* or *Cryptosporidium* with live weight in cattle (Ralston et al., 2003; Geurden et al., 2006; Castro-Hermida et al., 2007; Geurden et al., 2010a).

Clinical disease signs associated with protozoa infections (poor weight gain and diarrhoea) are most commonly described in young naive (î) livestock (Xiao, 1994; Olson et al., 1995; Nydam et al., 2001; Olson et al., 2004). In the present study, average growth rate
between the first marking sampling and the second sampling was lower in unweaned lambs which tested positive for Cryptosporidium.

Lambs on both farms positive for Cryptosporidium on the first marking sampling and the second sampling, had lighter live weights when compared to uninfected lambs (Table 9.3). Other factors which have impacts on live weight measurements, include lamb feed intake, gut fill, time off feed (Thompson et al., 1987; Warriss et al., 1987) and whether lambs were single or twin born (Kenyon et al., 2004; Corner et al., 2006; 2007; Hatcher et al., 2009). Lamb birth type (single or twin) was not recorded in the present study. Clinical signs of these protozoan infections (poor weight gain and diarrhoea) have been stated to be most common in young, ï livestock when infected with these protozoa (Xiao, 1994; Olson et al., 1995; Nydam et al., 2001; Olson et al., 2004) and this is possibly why significant impacts on live weight and growth rate were observed across the first marking sampling and the second sampling (lambs less than 2 months of age).

Diarrhoea is a common clinical sign associated with Cryptosporidium and Giardia infections in livestock, with other signs including poor performance, dehydration and abdominal pain (Olson et al., 1995; Fayer et al., 2000b; Ryan et al., 2005; Aloisio et al., 2006; Ozdal et al., 2009). Diarrhoea in sheep has been ascribed to many factors and is a multi-factorial condition caused by intestinal parasites, bacterial or viral pathogens, fungal endophytes, dietary composition and water absorption (Morley et al., 1976; Mitchell and Linklater, 1983; Taylor et al., 1993; Larsen et al., 1994; Eerens et al., 1998; Jacobson et al., 2009b). In the present study, there were significant associations observed between FCS and detection of Cryptosporidium in young lambs (2 months of age) at the second sampling and Cryptosporidium-positive lambs were 2.2 to 3.8 times more likely to have
FCSs in non-pellet form. This reinforces the belief that young lambs are more prone to suffer from diarrhoea associated with protozoa infection. *Giardia*-positive lambs on Farm A were 3.1 times more likely to have non-pelleted faeces on the final sampling, although this sampling occurred after mustering, handling and transport of lambs into lairage (pre-slaughter holding pens at the abattoir), and diarrhoea is typically one clinical sign associated with the elevated stress levels observed in lairage environments (Warriss *et al.*, 1989; Grandin, 1997; Hall *et al.*, 1999; Sotiraki *et al.*, 1999; Fisher *et al.*, 2010). Noticeably on both farms, there were only significant associations between the species/genotype of *Cryptosporidium* or *Giardia* with faecal attributes (FCS and breech fleece faecal soiling score). Lambs positive for *C. ubiquitum* or *Giardia duodenalis* assemblage A or A and E, had higher FCSs and further research is warranted to determine if species/genotype effects are significantly associated with production consequences.

Strongylid nematodes are the major internal parasites challenging sheep productivity and welfare worldwide and are the most common cause identified for diarrhoea outbreaks in lambs less than 12 months old (Mitchell and Linklater, 1983; Sargison, 2004; Broughan and Wall, 2007). Strongylid nematodes have been associated with reduced live weight, growth rate and BCS (Dargie and Allonby, 1975; Datta *et al.*, 1999; Macchi *et al.*, 2001; Sykes and Greer, 2003; Greer, 2008; Sutherland *et al.*, 2010), reduced carcase weight (Coop *et al.*, 1986; Sutherland *et al.*, 2010), reduced bone growth (Sykes *et al.*, 1977; Coop *et al.*, 1981) and mortalities (Dargie and Allonby, 1975). In the present study, lambs on each farm were grazed as a single flock on pastures that were contaminated with strongylid nematode larvae. All lambs on both farms were naturally exposed to strongylid larvae and consequently it was not possible to quantify the effect of larval challenge on growth rate, faecal consistency or carcase productivity. There was no correlation observed
between adjusted WEC with any carcase productivity, growth rate or live weight attributes, but higher WECs were associated with increased FCS, reduced FDM% and increased breech fleece faecal soiling score at several sampling occasions. This was consistent with other studies demonstrating the challenge with *T. circumcincta* and *T. colubriformis* impacted faecal consistency (Larsen and Anderson, 2000; Williams *et al.*, 2010c) and poor correlations of WEC with live weight and growth rate within flocks challenged with strongylid larvae (Eady *et al.*, 1998; Coop and Kyriazakis, 1999; Safari and Fogarty, 2003).

If impacts on carcase productivity and faecal attributes are consistent findings in grazing lambs, possible future research directions may include strategic targeted treatments or early vaccination of the lambs. Treatment of extensively managed lambs is problematic because repeated treatments are required and re-infection with *Cryptosporidium* and *Giardia* (oo)cysts from the pasture environment (soil, vegetation, faeces) is likely, because (oo)cysts are capable of surviving for extended periods in the faecal, soil or water environments (Robertson *et al.*, 1992; Fayer *et al.*, 1996; Carey *et al.*, 2004). Recent studies in young lambs and goat kids (approximately 2 weeks of age) showed that chemical treatments for cryptosporidiosis had no effect on live weight gain, only significantly reducing the incidence of diarrhoea and oocyst output (Giadinis *et al.*, 2007; 2008). However a study in housed Holstein calves, that were orally infected with *Giardia* trophozoites (two groups of 14), showed the group of calves that received a fenbendazole treatment had increased final live weights and increase growth rates, compared to untreated calves (Geurden *et al.*, 2010a).

Also worthy of note is that *C. jejuni* was isolated by PCR in less than 1% of lambs at slaughter in both flocks. Other studies have reported high prevalences of *C. jejuni* detected
in sheep faecal samples and also in their fleeces and carcases (Gill et al., 1998a; 1998b; Woldemariam et al., 2005; Garcia et al., 2010). *Campylobacter jejuni* is the leading cause of bacterial gastroenteritis in humans, whereby meat products are commonly assumed to be the original source (Olson et al., 2008; Ogden et al., 2009). Hence finding low point prevalences in lambs at slaughter, suggests that carcasses originating from these flocks represented a low risk for *Campylobacter*-related food poising to consumers. Further investigation is warranted to determine their prevalence and associations with lamb productivity, whilst examining possible sources of contamination.

**9.5 CONCLUSION**

*Cryptosporidium* and *Giardia* have been reported in sheep raised both under extensive conditions (grazing pastures at low-moderate stocking densities) and intensive conditions (housed), but the productivity consequences of infection on sheep meat production have not been well described. This study identified negative consequences for important carcase profit indicators (carcass weight and dressing percentage) associated with the identification of *Cryptosporidium* detection (both Farms A and B) and *Giardia* (Farm B only). Associations were also identified between these protozoa and reduced live weight and more loose faeces (diarrhoea) in lambs, but not at all sampling occasions. The quantifiable differences observed between lambs which tested either positive or negative for these protozoa, supports claims in other studies that these parasites limit productivity of small ruminants (Aloisio et al., 2006). Further investigation into the consequences between strongylid nematode and protozoa interactions on lamb production will need to be examined to determine the relative importance of these parasites. If protozoa are consistently found to be significantly impacting on productivity performances in a variety of different ruminant models, then further research is needed to determine if vaccines or
prophylactic treatments would be cost-effective for livestock enterprises to reduce production losses attributable to protozoa infections.
CHAPTER 10: COMPARING DIFFERENT STRONGYLID LARVAE CHALLENGES

DEVELOPMENT OF A MODIFIED MOLECULAR DIAGNOSTIC PROCEDURE FOR THE IDENTIFICATION AND QUANTIFICATION OF NATURALLY OCCURRING STRONGYLID LARVAE ON PASTURES


10.1 INTRODUCTION

Strategic management and control of strongylid parasites in commercial sheep enterprises is critical because of the income loss associated with reduced flock productivity (Sackett et al., 2006). The most economically important sheep strongylid genera in southern Australia are Trichostrongylus spp., Teladorsagia circumcincta and Haemonchus contortus and to a lesser extent Chabertia ovina, Oesophagostomum spp. and Nematodirus spp. (Besier and Love, 2003; Woodgate and Besier, 2010). Accurate and reliable species diagnosis and quantification of strongylid infections are both essential for the development of effective control programs (Coles et al., 2006; Woodgate and Besier, 2010). Providing estimates regarding number and species of larvae on pastures, along with species and worm burden estimates in lambs, are both important for the management and control of sheep parasites. Rapid identification of highly pathogenic strongylids from both lambs and pastures would aid grazing management, enhance the development of strategic
anthelmintic treatment programs, assist with monitoring resistance for anthelmintic efficacy trials and improve the surveillance of strongylids across different geographical regions.

Assessment of the number and species of strongylid larvae on pastures, is typically performed either by direct quantification of larvae recovered from pastures through pasture larvae counts, or by introducing worm-free ‘tracer’ sheep to a flock for a short period and then conducting faecal worm egg counts (WECs) or post-mortem total worm counts (Martin et al., 1990). Pasture larval counts are time consuming (7–10 days to perform), labour intensive and costly. The latter ‘tracer’ sheep method has the advantage of ‘tracer’ animals mimicking the grazing patterns of the resident flock. However, faecal sampling the ‘tracer’ animals is particularly difficult to perform in paddock, total worm counts require the sacrifice of sheep and not all larvae ingested by sheep develop into adults (Dobson et al., 1990b; Martin et al., 1990).

The diagnosis and/or quantification of parasitic infection by use of DNA-based tests is increasing (Hunt, 2011), with recent molecular diagnostic techniques utilised to detect strongylid species in sheep. These techniques involve genomic DNA extracted using either worm eggs that have been column-purified from faeces (Bott et al., 2009; Roeber et al., 2011) or extraction directly from unprocessed faeces (Chapters Five and Six). The DNA was then screened using previously developed strongylid specific-specific primers (Bott et al., 2009). Molecular diagnostic tests are routinely used for detection of water-borne parasites, including Cryptosporidium and Giardia.

In Chapters Four and Seven, livestock water source was seen as a potential risk factor for transmission of protozoan parasites and this was further investigated in this
study. Furthermore, similar molecular diagnostic methods as employed in Chapters Five, Six, Seven, Eight and Nine, were utilised to detect protozoan and strongylid worms.

10.1.1 AIMS AND HYPOTHESES

The aims of this study were:

1. To compare the impact of different natural strongylid larvae challenges on lamb flock productivity performances (carcase weight, dressing percentage, live weight, growth rate, BCS) and faecal attributes (FCS, breech fleece faecal soiling score and FDM%).
2. To develop and test a qPCR diagnostic method for the recovery, identification and quantification of strongylid larvae species from pasture.
3. To assess whether drinking water source is a potential source of Cryptosporidium or Giardia.
4. To utilise PCR and qPCR assays to detect multiple parasite species from genomic DNA extracted directly from lamb faeces and compare differences in internal parasite prevalences and species/genotypes between flocks.
5. To assess whether within flock analysis reveals if any strongylid or protozoa parasites are associated with reduced productivity performances and faecal attributes.

The hypotheses of this experiment were that:

1. Lambs exposed to a low natural strongylid L\textsubscript{3} challenge have higher dressing percentages and carcase weights at slaughter, increased growth and body condition
(live weight, growth rate and BCS) and firmer faeces, compared with lambs exposed to a higher standard strongylid L₃ challenge.

2. The qPCR diagnostic method accurately and reliably detects different strongylid species from DNA extracted from material recovered from pastures.

3. Cryptosporidium and Giardia are present in livestock water sources.

4. Lambs exposed to lower levels of strongylid larvae have lower parasite prevalences when compare to lambs exposed to higher strongylid larvae levels.

5. Protozoan parasites are associated with lower carcase weight and dressing percentage, with no difference in their impact observed between high and low larval challenges.

**10.2 MATERIALS AND METHODS**

**10.2.1 STUDY SITES, ANIMALS, ANTHELMINTIC TREATMENTS AND EXPERIMENTAL PROTOCOL**

**10.2.1.1 Study site**

This experiment was approved and supervised by the Murdoch University Animal Ethics Committee (permit R2236/09). The two sheep flocks in this study were located ~350–400km south of Perth in separate paddocks on the same property. The farm was in Frankland (32.55° S, 116.87° E), which is ~360km south-east of Perth, in a region of Western Australia that experiences a Mediterranean environment (hot, dry summers and cool, wet winters) (Hill *et al.*, 2004; Moeller *et al.*, 2008). Average annual rainfall for this property was ~550mm.
The first paddock (Paddock S) was 24 hectares and had a ‘standard’ strongylid larvae challenge (Flock S), whereby the paddock was grazed by pregnant ewes and lambs the previous year (2010). Merino ewes in this present study (n=390 ~6 years old) grazed the paddock from February 2011 onwards (following joining/mating with Suffolk rams).

The second paddock (Paddock L) was 18 hectares and was not located directly adjacent to Paddock S (~2km away). Paddock L was managed to minimise the natural strongylid larvae challenge for 2011 (low natural strongylid larvae challenge). Briefly, in 2009 this paddock was used for cropping with a cereal grain (barley) and adult wethers grazed the crop residues during November and December. In 2010, the paddock was left un-grazed to allow pasture regeneration without livestock. In early July 2011, the flock grazing Paddock S was split, whereby 180 of the 390 pregnant ewes on Paddock S were randomly selected, treated with an anthelmintic (2.5 mg monepantel/kg of live weight Monepantel, Zolvix, Novartis Australia), administered according to the heaviest ewe live weight) and introduced to Paddock L.

The dry sheep equivalents per hectare (DSE/ha) 2011 winter stocking rates for Flocks S and L were 21.1 DSE/ha and 20.7 DSE/ha, respectively (McLaren, 1997). No cattle or goats were present on the property. The major plant species present in both paddocks consisted primarily of annual rye-grass (Lolium spp.) and subterranean clover (Trifolium subterraneum). Feed on offer (FOO) was calculated for each paddock (Table 10.1 and Figure 10.1) (Australian Wool Innovation, 2007). Pasture quality was analysed by FeedTest (Australian Wool Testing Authority Ltd - Agrifood Technology, Victoria Australia) using near infrared spectroscopy and results are shown in Table 10.2.
**Table 10.1:** Lamb age and feed on offer (FOO, kg of dry matter/hectare) on eight faecal sampling occasions.

<table>
<thead>
<tr>
<th>Sampling occasion</th>
<th>Lamb age</th>
<th>Study day</th>
<th>Date</th>
<th>Paddock S</th>
<th></th>
<th>Paddock L</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ewes/lambs sampled (n)</td>
<td>FOO</td>
<td>Ewes/lambs sampled (n)</td>
<td>FOO</td>
</tr>
<tr>
<td>16 weeks pre-partum</td>
<td>-</td>
<td>-155</td>
<td>March 22nd 2011</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2 weeks pre-partum *</td>
<td>-</td>
<td>- 49</td>
<td>July 6th 2011</td>
<td>50</td>
<td>2500</td>
<td>50</td>
<td>2200</td>
</tr>
<tr>
<td>4 weeks post-partum</td>
<td>-</td>
<td>0</td>
<td>August 24th 2011</td>
<td>50</td>
<td>2800</td>
<td>50</td>
<td>1900</td>
</tr>
<tr>
<td>Sampling 1</td>
<td>2-6 weeks</td>
<td>0</td>
<td>August 24th 2011</td>
<td>102</td>
<td>2800</td>
<td>101</td>
<td>1900</td>
</tr>
<tr>
<td>Sampling 2</td>
<td>2-3 months</td>
<td>42</td>
<td>October 5th 2011</td>
<td>102</td>
<td>3600</td>
<td>101</td>
<td>2300</td>
</tr>
<tr>
<td>Sampling 3 *</td>
<td>3-4 months</td>
<td>84</td>
<td>November 16th 2011</td>
<td>102</td>
<td>2900</td>
<td>101</td>
<td>1800</td>
</tr>
<tr>
<td>Sampling 4</td>
<td>4-5 months</td>
<td>117</td>
<td>December 19th 2011</td>
<td>102</td>
<td>-</td>
<td>101</td>
<td>-</td>
</tr>
<tr>
<td>Sampling 5</td>
<td>5-6 months</td>
<td>148</td>
<td>January 19th 2012</td>
<td>102</td>
<td>-</td>
<td>101</td>
<td>-</td>
</tr>
</tbody>
</table>

* Indicates one week prior to this sampling, 180 pregnant ewes were randomly selected from the combined flock and were treated with an anthelmintic (Monepantel, Zolvix), before introduced onto Paddock L.

* After this sampling received an anthelmintic treatment.

**Table 10.2:** Chemical analysis of pasture quality.

<table>
<thead>
<tr>
<th>Feed component</th>
<th>Paddock S</th>
<th></th>
<th>Paddock L</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sampling 1</td>
<td>Sampling 2</td>
<td>Sampling 3</td>
<td>Sampling 1</td>
</tr>
<tr>
<td>Dry matter (DM) %</td>
<td>12.9</td>
<td>13.4</td>
<td>18.6</td>
<td>7.7</td>
</tr>
<tr>
<td>Moisture %</td>
<td>87.1</td>
<td>86.6</td>
<td>81.4</td>
<td>92.3</td>
</tr>
<tr>
<td>Crude protein (% of DM)</td>
<td>26.1</td>
<td>27.1</td>
<td>19.4</td>
<td>30.2</td>
</tr>
<tr>
<td>Neutral detergent fibre (% of DM)</td>
<td>34.9</td>
<td>35.4</td>
<td>49.3</td>
<td>37.8</td>
</tr>
<tr>
<td>Digestibility (% of DM)</td>
<td>68.6</td>
<td>67.5</td>
<td>57.1</td>
<td>71.8</td>
</tr>
<tr>
<td>Metabolisable energy (MJ/kg DM)</td>
<td>10.9</td>
<td>10.7</td>
<td>8.2</td>
<td>11.6</td>
</tr>
</tbody>
</table>

Pasture quality was analysed by FeedTest (Australian Wool Testing Authority Ltd - Agrifood Technology, Victoria Australia) using near infrared technology.

Winter stocking rates for Paddocks S and L were 21.1 DSE/ha and 20.7 DSE/ha, respectively.
### Table 10.1

<table>
<thead>
<tr>
<th>Sampling occasions</th>
<th>March 22nd</th>
<th>April 23rd</th>
<th>May 25th</th>
<th>June 15th</th>
<th>July 6th</th>
<th>August 23rd</th>
<th>September 19th</th>
<th>October 6th</th>
<th>November 15th</th>
</tr>
</thead>
<tbody>
<tr>
<td>(normal larval challenge)</td>
<td>Size</td>
<td>24 hectares</td>
<td>FOO 800 kg DM/Ha</td>
<td>FOO 1300 kg DM/Ha</td>
<td>FOO 2000 kg DM/Ha</td>
<td>FOO 2500 kg DM/Ha</td>
<td>FOO 2800 kg DM/Ha</td>
<td>FOO 3200 kg DM/Ha</td>
<td>FOO 3600 kg DM/Ha</td>
</tr>
<tr>
<td>(low larval challenge)</td>
<td>Size</td>
<td>18 Hectares</td>
<td>FOO 500 kg DM/Ha</td>
<td>FOO 900 kg DM/Ha</td>
<td>FOO 1700 kg DM/Ha</td>
<td>FOO 2200 kg DM/Ha</td>
<td>FOO 1900 kg DM/Ha</td>
<td>FOO 2100 kg DM/Ha</td>
<td>FOO 2300 kg DM/Ha</td>
</tr>
</tbody>
</table>

**Figure 10.1**: Illustrations of varying feed on offer (FOO) at the nine different pasture samplings.
10.2.1.2 Animals, sample collection and measurements

The pregnant Merino ewes were sampled 16 weeks pre-partum (March 2011, before flock split), 2 weeks pre-partum (July 2011; one week after the combined flock was split) and 4 weeks post-partum (August 2011) (Table 10.1). A total of 50 faecal samples were collected from each flock (S and L) at all these sampling occasions, except for the first sampling (16 weeks pre-partum) when 100 faecal samples were collected from the flock (n=390), before it was split into two separate flocks (Table 10.1).

Each flock of lambs was raised on a single paddock (S or L). Following lambing, 102 and 101 female lambs from Flocks S and L respectively, were randomly selected and identified with both a numbered and a radio-frequency ear tag at marking. Faeces were collected directly from the rectum using fresh latex gloves to prevent cross contamination between faecal samples. Lambs were yarded for weighing, assessment of breech fleece faecal soiling and faecal sample collection on five separate sampling occasions (Table 10.1).

Faecal samples were collected from lambs during lairage at the final sampling, ~12 hours before the lambs were slaughtered. A total 102 and 101 lambs from Paddock S and L respectively, were sampled on all five sampling occasions. All faecal samples were placed in individually labelled, airtight 70mL containers and transported to the laboratory within 6 hours of collection. Faecal samples were stored at 2–4°C and genomic DNA was extracted from each sample within ~7 days of collection. The transport and storage practices utilised in this study were consistent with other similar studies that used PCR to detect these parasites (Yang et al., 2009; Ng et al., 2010a; 2010b; Robertson et al., 2010).

Lamb live weight was recorded at all five samplings. Body condition score was recorded at the last four samplings by using a scale that ranged from 1 (very thin,
emaciated) to 5 (excessively fat) (Sutherland et al., 2010). Faecal attributes were measured at all samplings. Faecal consistency score (FCS) was measured using a scale of 1 (hard, dry pellet) to 5 (liquid/fluid diarrhoea), previously described (Greeff and Karlsson, 1997; Le Jambre et al., 2007). Faecal dry matter percentage (FDM%) was measured on fresh faeces that had been stored in air tight containers for approximately 24 hours using methods described by the Association of Official Analytical Chemists (AOAC, 1997). Breech fleece faecal soiling score was recorded at the final four samplings (prior to crutching; removal of faecal soiled fleece from the breech area of lambs). Breech faecal soiling was measured using a scale of 1 (no evidence of breech fleece faecal soiling) to 5 (very severe breech fleece faecal soiling extending down the hind legs to, or below the hocks). A graphical illustration of these breech fleece faecal soiling scores is available in the Australian Wool Industry’s Visual Sheep Score booklet (Australian Wool Innovation et al., 2007).

Lambs were slaughtered at a commercial abattoir. Standard hot carcase weight (HCW) was recorded for all lambs. Carcase fat score was recorded on a scale of 0 (very lean) to 5 (excessively fat) (Hopkins, 1992).

A 10L water sample was collected from each flock’s water source on the first three samplings. Water was supplied ad libitum via a clay dam (Flock S) and trough water sourced from a dam (Flock L).

10.2.1.3 Lamb management and anthelmintic treatment

Lambs were treated with an anthelmintic (2mL/10kg of animal live weight [0.2mg abamectin/kg of live weight] Paramectin Mineralised, Jurox, Australia, administered according to the heaviest lamb live weight) following the third sampling (Table 10.1).
Lambs were weaned from their mothers back onto their respective trial paddocks (S or L) also after the third sampling. Approximately 100 g/head/day of a supplementary feed grain mixture (comprising 35% lupins and 65% oats) was given to each lamb flock following weaning. Lambs were shorn 14 days prior to the fourth sampling. Lambs were held off feed overnight before being weighed and then transported to a commercial abattoir for slaughter.

10.2.3 FAECAL WORM EGG COUNTS

Faecal worm egg counts (WECs) were performed within 2 days of faecal collection using a modified McMaster technique (Lyndal-Murphy, 1993). Two grams of faeces were used from each sample and each egg counted represented 50 epg of faeces. An insufficient quantity of faecal material was collected in some samples at the first sampling occasion and as a result some WEC data were missing for some animals at this sampling. Following counting of strongylid worm eggs, a search for coccidia oocysts was conducted in one quarter of a counting chamber to determine if the lamb/sheep was infected with *Eimeria* (Lyndal-Murphy, 1993).

10.2.4 PASTURE COLLECTION AND PASTURE LARVAL COUNTS

Pasture grazed from Paddocks S and L on the Frankland property was collected from 200 sites at roughly equal distances along a W-shaped transect (Taylor, 1939; Martin *et al.*, 1990). Four “plucks” of pasture were collected at each site from in front, behind, left and right of the sampler. Duplicate pasture samples (two sets of four “plucks”) were collected at the sampling sites of both paddocks. Where possible, “plucks” did not include roots, soil or faeces. Pasture was kept at 2–4°C until processed. Pasture samples were collected on nine different occasions (Table 10.3). Pasture larval counts were conducted by
Animal Health Laboratories, at the Department of Agriculture and Food (Albany, Western Australia) using the method described in the Animal Health Laboratory Research Methods (Animal Health Laboratories, 2005b) and modified according to Martin et al. (1990). This technique detects all three strongylid nematode larval stages.

10.2.5 MODIFIED STRONGYLID LARVAE RECOVERY PROCEDURE

A modified method was developed to detect and quantify different strongylid larvae species recovered from pasture. This procedure involved collecting pasture/grass samples as described in the ‘pasture larvae counts’ section (Taylor, 1939; Martin et al., 1990). The sample was emptied into a 300µm nylon cylindrical mesh bag and the top of the nylon bag was then secured tightly using a cable tie. The bag was then placed inside the pasture washer (Figure 10.2) and a total of 60L of water was added to the pasture washer, along with 3g of Pyroneg detergent (Johnson Diversey, Manukau, New Zealand). The nylon bag had an internal steel frame with a T-piece handle at the top, which allowed the sample to be gently agitated each hour for ~60 seconds. The submerged pasture sample was soaked for 6 hours.

After soaking, the water was released using a ball valve situated at the bottom of the washer. The water passed through 300mm of 100mm PVC pipe containing two stainless steel sieves, with apertures of 75µm and 25 µm, respectively. Once all the water had been drained through the sieve, this 300mm bottom section of pipe holding the sieves was removed and using a fine jet of distilled water, the material caught by the sieves was washed into a 50mL centrifuge tube. The pasture washer was refilled and drained a second time. The pipe holding the sieves was again removed, with the material caught washed from the sieve using a fine jet of distilled water into the same 50mL centrifuge.
tube. The 50mL centrifuge tube was then centrifuged at 1150 \( g \) for 15 minutes. The supernatant was removed without disturbing the centrifuged pellet, which was separated into 10 separate equal sub-samples in separate 1.5mL Eppendorf tubes. The 10 tubes were centrifuged at 10000 \( g \) for 1 minute and any remaining fluid was removed following centrifugation. Genomic DNA was extracted from each pellet (weighing ~300mg) with the Power Soil DNA Isolation Kit (MO BIO Laboratories, Inc.; 2746 Loker Avenue West; Carlsbad, CA 92010) using the modified protocol (Chapter Five). A total of 180 genomic DNA sub-samples were generated; 10 separate sub-samples yielded from each of the two paddocks, at the nine separate pasture sampling occasions.

A spike analysis was conducted, whereby pasture/grass samples were collected as described in the ‘pasture larvae counts’ section (Taylor, 1939; Martin et al., 1990) from areas without any grazing livestock. These samples were then spiked with known \( L_3 \) quantities \( (n = 8, 40, 80, 200, 400, 1000) \) of either \( T. \text{circumcincta} \) (sample code OcNB1.2) or \( T. \text{colubriformis} \) (sample code Tc14). Following pasture washing and filtration (using the modified strongylid larvae recovery procedure detailed above), genomic DNA was extracted with Power Soil DNA Isolation Kits (MO BIO Laboratories, Inc.; 2746 Loker Avenue West; Carlsbad, CA 92010) using the modified protocol (Chapter Five). Pasture dry matter percentage was calculated following larvae recovery to determine the number of larvae/kg of pasture dry matter.
Figure 10.2: Graphical diagram of the pasture washer utilised to recover strongylid larvae for the modified strongylid larvae recovery procedure.
10.2.6 DNA EXTRACTION

A total of 1315 faecal samples (300 from pregnant ewes and 1015 from female lambs) were collected. A sub-sample was taken from the centre of each faecal sample. Sub-samples were weighed (250–300mg) and had genomic DNA extracted using the Power Soil DNA Isolation Kit (MO BIO Laboratories, Inc.; 2746 Loker Avenue West; Carlsbad, CA 92010). Minor modifications to the manufacturer’s protocol were made and have been detailed in previous studies (Chapters Five and Seven). After elution, DNA was stored at –20°C until use. Negative controls (known negative faecal sample) and positive controls (known negative faecal samples spiked with either Cryptosporidium or Giardia (oo)cysts, along with a 100µL suspension containing one of the five strongylid species [T. circumcincta, Trichostrongylus spp., H. contortus, C. ovina and Oesophagostomum spp.]) were produced.

Filtration of livestock drinking water was performed using Envirochek filters (Pall Life Sciences, New South Wales, Australia), in accordance with the manufacturer’s instructions, except for some slight modifications as described by Wohlsen et al., (2004). The eluate was subjected to the same genomic DNA extraction method detailed above.

10.2.7 STRONGYLID SPECIES qPCR AMPLIFICATION

The qPCR assays were conducted on a Rotor-Gene6000 Cycler (Qiagen, Hilden, Germany). Faecal and pasture genomic DNA extracts were screened using primers, protocol and thermocycling conditions for the strongylid species; Trichostrongylus spp., T. circumcincta and H. contortus, C. ovina and Oesophagostomum venulosum (Bott et al., 2009). For any samples that were McMaster WEC flotation positive (≥50 epg) and PCR negative or pasture larval count positive (>0 larvae/kg of pasture dry matter) and PCR
negative, five separate aliquots (10 µL) of the sample were spiked with 1 µL purified DNA from each of the five strongylid species (T. circumcincta, Trichostrongylus spp., C. ovina, O. venulosum and H. contortus). A 1 µL aliquot from each of the spiked 15 µL mixtures was then re-screened using qPCR assays to test for inhibition.

The cycle number at which the fluorescence threshold was exceeded (Cq) for each sample was established by setting threshold lines and calculating the intersection with each of the sample curves. Samples that crossed the threshold before 40 cycles were classified as positive and any samples that didn’t cross the threshold after 40 cycles were classified as negative. Positive controls and negative controls (no DNA), were included in each run (Bustin et al., 2009).

Using the modified Power Soil DNA Isolation Kit modified protocol (Chapter Five), genomic DNA was extracted from 250-300 mg of pasture samples spiked with known numbers of strongylid L3 (n = 8, 40, 80, 200, 400, 1000) containing whole L3 from only one strongylid species (either T. colubriformis or T. circumcincta). DNA concentrations were determined using the NanoDrop ND-1000 Spectrophotometer (Thermo Scientific). Positive control samples were serially diluted from 10^5 pg genomic DNA/µL to 10,000, 1000, 100, 50, 10, 5, 2, 1 and 0.1 pg/µL to determine the minimum amount of genomic DNA required for successful qPCR amplification.

**10.2.8 PCR AMPLIFICATION**

Pasture larval DNA extracts that were qPCR positive for any strongylid species were re-screened using species-specific primers (Bott et al., 2009) in conventional PCR assays (Chapters Five and Six).
All samples (faeces and pasture DNA) were screened for *Cryptosporidium* and *Giardia*. A two-step nested PCR protocol was used to amplify the 18S rRNA locus of *Cryptosporidium* previously described by Ryan *et al.* (2003), producing an ~540bp product. All *Cryptosporidium* positive samples were also screened using a different two-step nested PCR protocol, conducted to amplify a product of ~830bp at the actin gene of *Cryptosporidium*, as described by Ng *et al.*, (2006), to identify mixed infections (i.e. one species/genotype amplified at the 18S locus and a different species/genotype identified at the actin locus).

All samples were screened for *Giardia* at the *gdh* (glutamate dehydrogenase) gene as previously described by Read *et al.*, (2004), producing an ~480bp product. Samples identified as positive for *Giardia* at the *gdh* gene by PCR, were screened at the β-giardin gene with a two-step nested PCR protocol. The primary PCR reaction was performed as described by Cacciò *et al.*, (2002), producing an ~735bp product and the secondary PCR reaction amplified an ~511bp product using primers and PCR conditions previously described by Lalle *et al.*, (2005). Positive samples were screened with nested PCRs at each of these two loci (*gdh* and β-giardin), to confirm the assemblage(s) and whether mixed infections existed.

### 10.2.9 SEQUENCE ANALYSIS

Positive *Cryptosporidium* (18S rRNA), *Giardia* (*gdh*), and strongylid (ITS-2 rDNA) PCR products isolated were purified using an UltraClean™ DNA Purification Kit (MO BIO Laboratories, Inc.; 2746 Loker Avenue West; Carlsbad, CA 92010) and sequenced using an ABI Prism Terminator Cycle Sequencing Kit (Applied Biosystems) on an Applied
Biosystem 3730 DNA Analyzer. Sequence searches were conducted using BLAST\(^4\) and nucleotide sequences were analysed using Chromas Lite version 2.0\(^5\). Nucleotide sequences were analysed using Chromas Lite version 2.0 (http://www.technelysium.com.au) and alignment confirmed with reference isolates from GenBank using Clustal W (http://www.clustalw.genome.jp). Strongylid sequences from genomic DNA extracted from material recovered using the modified strongylid larvae recovery procedure, were aligned with reference genotypes to confirm positive identification for *H. contortus*, *T. circumcincta*, *Trichostrongylus axei*, *T. colubriformis*, *C. ovina*, *O. venulosum* or *O. columbianum* (GenBank AJ57746.1, AJ577463.1, AY439026.1, EF427624, AY439021.1, Y10790.1 and AJ006150, respectively) using Clustal W\(^6\).

Phylogenetic trees were constructed for *Cryptosporidium* isolates at 18S rRNA and actin loci, for *Giardia* isolates at the *gdh* and \(\beta\)-giardin loci and also *Eimeria* isolates at the 18S rRNA locus, with additional isolates obtained from GenBank. Distance estimation was performed, based on evolutionary distance calculations with the Kimura 2-parameter model and grouped firstly using TREECON software (Van de Peer and De Wachter, 1994) to conduct Neighbour-Joining analysis and secondly using Mega 5 software to conduct maximum-parsimony analysis (Kumar *et al.*, 2008). The confidence of groupings from both analyses was assessed by bootstrapping, using 1000 replicates. A percentage bootstrap support of >50% was used for each phylogenetic tree constructed.


10.2.10 STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS Statistics 17.0 (Statistical Package for the Social Sciences) for Windows (SPSS inc. Chicago, USA). Parasite prevalences (including 95% confidence intervals [CI]) at each sampling occasion and overall (lambs positive for a parasite on one or more sampling occasions) were calculated using the exact binomial method (Thrusfield, 2007). The WEC data were categorised as positive (WEC ≥50 epg) or negative (no strongylid eggs detected). To assess the level of agreement between the McMaster WEC and qPCR results, along with pasture larval counts and qPCR results, Cohen’s Kappa (κ) statistic was calculated at each sampling occasion, overall for each farm (all five samplings combined) and overall for the entire study (both farms combined). Categorical data were analysed to test the level of agreement between WEC (WEC≥50 epg) and qPCR results (positive vs. negative), along with pasture larvae counts (positive if larvae were detected and negative if no larvae detected) and qPCR results.

Categorical data between the two flocks (parasite prevalences) were compared using Pearson’s chi squared or Fisher’s exact two-sided test for independence. Variances were tested using Levene’s test of equality for error variance.

McMaster WEC data were adjusted for that consistency associated with normally formed faeces (FCS=1) according to the following equation described by Le Jambre et al., (2007):

\[
Adjusted\ WEC = \frac{Raw\ WEC}{(34.21 - 5.15 \times FCS)} \times 29.06
\]

Adjusted WEC data were transformed using \( \log_{10}(\text{adjusted WEC}+25) \) to stabilise variances prior to statistical analysis (Dobson et al., 2009). Pasture larvae counts (number
of larvae [per strongylid species]/kg of pasture dry matter) was transformed using \( \log_{10}(\text{number of larvae [per species]}) \) prior to comparison with qPCR \( C_q \) values. Correlation between qPCR \( C_q \) values and adjusted, transformed WEC from lambs only positive for one strongylid species (single strongylid infection), was estimated by linear regression using a Pearson correlation (two-tailed test for significance). Correlation between pasture qPCR \( C_q \) values and pasture larvae count results was estimated by linear regression using a Pearson correlation two-tailed test for significance, with \textit{Trichostrongylus} spp. and \textit{T. circumcincta} results were analysed separately from one another.

Lamb growth rate was expressed between sequential sampling occasions as both grams gained/day (g gained/day) and percentage liveweight change between sampling occasions (% gained/day).

Production (HCW, dressing percentage, GR knife fat depth, live weight, growth rate and BCS) and faecal (FCS, FDM\% and breech fleece faecal soiling score) attributes were normally distributed and the variances were not significantly different (Levene’s test \( P>0.05 \)) so between-flock analyses for these parameters were performed using ANOVA. The distributions of adjusted, log-transformed WECs were non-normal and the variances were significantly different, therefore adjusted WECs from each flock were compared using a two-tailed non-parametric Mann-Whitney \( U \) test.

Within flock analyses were also conducted (each flock analysed separately to the other). Lambs were classified as negative (never tested positive at any sampling occasion) or positive (tested positive on at least one sampling occasion) for \textit{Cryptosporidium} and \textit{Giardia}. Positive-lambs were sub-categorised as positive once (positive on one occasion) or repeat positive (infected on more than one occasion). Lambs were classified according
to the number of sampling occasions at which they tested positive for the above parasites. Overall prevalences were calculated for lambs that were classified as positive for each protozoa genera and strongylid-positive by using the exact binomial method (Thrusfield, 2007).

General linear model analyses were performed for carcase attributes HCW, dressing percentage and fat score as dependent variables. Positive/negative parasite classification and the number of occasions when lambs tested positive for Cryptosporidium and Giardia were included as independent variables. Mean, log-transformed adjusted WEC (across all sampling occasions for the respective farm) was included as a covariate. Differences in the mean measurements for carcase attributes were analysed only on the lambs sampled at all five sampling occasions using the least significant differences (LSD) post-hoc test.

Correlation between adjusted, log-transformed WECs with HCW, dressing percentage and fat score were analysed separately for each farm using linear regression with an ANOVA test for significance. Where significant correlations were identified, the difference across the WEC range observed was estimated using the linear regression function.

10.3 RESULTS

10.3.1 INTERNAL PARASITES PREVALENCES IN EWES AND LAMBS

Both strongylid and protozoan species were detected in faecal samples from pregnant ewes (Table 10.3). There were no significant differences in protozoan prevalences between the two pregnant ewe flocks, except at 4 weeks post-partum, when
Flock S had a higher *Eimeria* prevalence compared to Flock L (P<0.001). For the strongylid species, Flock S had higher *T. circumcincta* and *Trichostrongylus* spp. prevalences than Flock L at both samplings; 2 weeks pre-partum and 4 weeks post-partum (P<0.001). Flock S had higher *C. ovina* and *Oesophagostomum* spp. prevalences than Flock L at 2 weeks pre-partum (P<0.05). Flock S had higher adjusted average WECs than Flock L at both 2 weeks pre-partum and 4 weeks post-partum (P<0.001) (Table 10.3).
Table 10.3: Internal parasite flock prevalences by qPCR (95% CI), WEC and FCS average and ranges for the pregnant ewe flocks.

<table>
<thead>
<tr>
<th>Time in pregnancy</th>
<th>Combined flock</th>
<th>Flock S</th>
<th>Flock L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnant ewes sampled (n)</td>
<td>100</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Flock size</td>
<td>380</td>
<td>210</td>
<td>210</td>
</tr>
<tr>
<td>Parasite flock prevalence</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryptosporidium</td>
<td>18.0</td>
<td>20.0</td>
<td>18.0</td>
</tr>
<tr>
<td>(11.0, 26.9)</td>
<td>(10.0, 33.7)</td>
<td>(8.6, 31.4)</td>
<td>(5.8, 27.6)</td>
</tr>
<tr>
<td>Giardia</td>
<td>28.0</td>
<td>22.0</td>
<td>30.0</td>
</tr>
<tr>
<td>(19.5, 39.5)</td>
<td>(11.5, 36.0)</td>
<td>(17.9, 44.6)</td>
<td>(7.2, 29.1)</td>
</tr>
<tr>
<td>Eimeria</td>
<td>14.0</td>
<td>18.0</td>
<td>34.0</td>
</tr>
<tr>
<td>(7.9, 22.4)</td>
<td>(8.6, 31.4)</td>
<td>(21.2, 48.8)</td>
<td>(8.6, 31.4)</td>
</tr>
<tr>
<td>T. circumcincta</td>
<td>12.0</td>
<td>34.0</td>
<td>46.0</td>
</tr>
<tr>
<td>(6.4, 20.0)</td>
<td>(21.2, 48.8)</td>
<td>(31.8, 60.7)</td>
<td>(0.0, 7.1)</td>
</tr>
<tr>
<td>Trichostrongylus spp.</td>
<td>22.0</td>
<td>32.0</td>
<td>68.0</td>
</tr>
<tr>
<td>(14.3, 31.4)</td>
<td>(19.5, 46.7)</td>
<td>(53.3, 80.5)</td>
<td>(0.0, 7.1)</td>
</tr>
<tr>
<td>H. contortus</td>
<td>0.0</td>
<td>6.0</td>
<td>8.0</td>
</tr>
<tr>
<td>(0.0, 3.6)</td>
<td>(1.3, 16.5)</td>
<td>(2.2, 19.2)</td>
<td>(0.0, 7.1)</td>
</tr>
<tr>
<td>C. ovina</td>
<td>9.0</td>
<td>18.0</td>
<td>14.0</td>
</tr>
<tr>
<td>(4.2, 16.4)</td>
<td>(8.6, 31.4)</td>
<td>(5.8, 26.7)</td>
<td>(0.0, 7.1)</td>
</tr>
<tr>
<td>Oesophagostomum spp.</td>
<td>4.0</td>
<td>10.0</td>
<td>6.0</td>
</tr>
<tr>
<td>(1.1, 9.9)</td>
<td>(3.3, 21.8)</td>
<td>(1.3, 16.5)</td>
<td>(0.0, 7.1)</td>
</tr>
<tr>
<td>Adjusted average flock WEC ± S.E.M b epg (range)</td>
<td>83 ± 21</td>
<td>178 ± 32.1</td>
<td>363 ± 48</td>
</tr>
<tr>
<td>(0 – 1067)</td>
<td>(0 – 775)</td>
<td>(0 – 1346)</td>
<td>(0)</td>
</tr>
<tr>
<td>Average FCS (range)</td>
<td>1.7 (1 – 4)</td>
<td>3.2 (1 – 4.5)</td>
<td>3.1 (1 – 5)</td>
</tr>
</tbody>
</table>

Note: the combined flock was separated into two different flocks (S and L) ~1 week prior to the sampling 2 weeks pre-partum.

* Indicates one week prior to this sampling, 180 pregnant ewes were randomly selected from the combined flock and treated with an anthelmintic (Monepantel, Zolvix), before introduced onto Paddock L.

b Faecal worm egg counts (WECs) were adjusted for faecal consistency score (FCS).
Overall and point prevalences for strongylid and protozoan parasites detected in each lamb flock are presented in Table 10.4. Any significant differences between the parasite prevalences for each flock are also displayed in this table.

Adjusted WEC at each sampling for the two lamb flocks are shown in Table 10.5. Flock S had higher adjusted WEC than Flock L at the first, second and final sampling occasions (Table 10.5).

The number of samplings that lambs from each flock tested qPCR positive for each strongylid species are presented in Figure 10.3 (A) and the proportion of lambs with different numbers of strongylid species detected in Figure 10.3 (B). The correlation between log-transformed WEC and qPCR Cq values for lambs positive for only one strongylid species was weak ($r^2=0.25$) and is presented in Figure 10.3 (C)
Table 10.4: Internal parasite prevalences (95% CI), along with levels of agreement between McMaster WEC and qPCR results.

<table>
<thead>
<tr>
<th>Lamb age</th>
<th>2-6 weeks</th>
<th>2-3 months</th>
<th>3-4 months</th>
<th>4-5 months</th>
<th>6-7 months</th>
<th>Overall Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>42</td>
<td>84</td>
<td>117</td>
<td>148</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Flock S</td>
<td>Flock L</td>
<td>Flock S</td>
<td>Flock L</td>
<td>Flock S</td>
<td>Flock L</td>
</tr>
<tr>
<td>Identified lambs sampled (n)</td>
<td>102</td>
<td>101</td>
<td>102</td>
<td>101</td>
<td>102</td>
<td>101</td>
</tr>
<tr>
<td>Parasite prevalence (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryptosporidium</td>
<td>11.8^</td>
<td>7.9^</td>
<td>25.5^</td>
<td>13.9^</td>
<td>18.6^</td>
<td>18.8^</td>
</tr>
<tr>
<td>(6.2, 19.6)</td>
<td>(3.5, 15.0)</td>
<td>(17.4, 35.1)</td>
<td>(7.8, 22.2)</td>
<td>(11.6, 27.6)</td>
<td>(11.7, 27.8)</td>
<td>(10.0, 25.3)</td>
</tr>
<tr>
<td>Giardia</td>
<td>7.8^</td>
<td>8.9^</td>
<td>16.7^</td>
<td>11.9^</td>
<td>18.6^</td>
<td>7.9^</td>
</tr>
<tr>
<td>(3.4, 14.9)</td>
<td>(4.2, 16.2)</td>
<td>(10.0, 25.3)</td>
<td>(6.3, 19.8)</td>
<td>(11.6, 27.6)</td>
<td>(3.5, 15.0)</td>
<td>(8.5, 23.1)</td>
</tr>
<tr>
<td>Eimeria</td>
<td>7.8^</td>
<td>8.9^</td>
<td>8.8^</td>
<td>8.9^</td>
<td>10.8^</td>
<td>7.9^</td>
</tr>
<tr>
<td>(3.4, 14.9)</td>
<td>(4.2, 16.2)</td>
<td>(4.1, 16.1)</td>
<td>(4.2, 16.2)</td>
<td>(5.5, 18.5)</td>
<td>(3.5, 15.0)</td>
<td>(2.8, 13.6)</td>
</tr>
<tr>
<td>T. circumcincta</td>
<td>9.8^</td>
<td>5.0^</td>
<td>46.1^</td>
<td>5.9^</td>
<td>78.4^</td>
<td>21.8^</td>
</tr>
<tr>
<td>(4.8, 17.3)</td>
<td>(1.6, 11.2)</td>
<td>(36.2, 56.2)</td>
<td>(2.2, 12.5)</td>
<td>(69.2, 86.0)</td>
<td>(14.2, 31.1)</td>
<td>(9.2, 24.2)</td>
</tr>
<tr>
<td>Trichostrongylus spp.</td>
<td>7.8^</td>
<td>10.9^</td>
<td>56.9^</td>
<td>8.9^</td>
<td>62.8^</td>
<td>31.7^</td>
</tr>
<tr>
<td>(3.4, 14.9)</td>
<td>(5.6, 18.7)</td>
<td>(46.7, 66.6)</td>
<td>(4.2, 16.2)</td>
<td>(52.6, 72.1)</td>
<td>(22.8, 41.7)</td>
<td>(3.4, 14.9)</td>
</tr>
<tr>
<td>H. contortus</td>
<td>0.0^</td>
<td>3.0^</td>
<td>49.0^</td>
<td>2.0^</td>
<td>46.1^</td>
<td>22.8^</td>
</tr>
<tr>
<td>(0.0, 3.6)</td>
<td>(0.6, 8.4)</td>
<td>(39.0, 59.1)</td>
<td>(0.2, 7.0)</td>
<td>(36.2, 56.2)</td>
<td>(15.0, 32.2)</td>
<td>(1.1, 9.7)</td>
</tr>
<tr>
<td>C. ovina</td>
<td>8.9^</td>
<td>3.0^</td>
<td>14.7^</td>
<td>8.9^</td>
<td>12.8^</td>
<td>25.7^</td>
</tr>
<tr>
<td>(4.2, 16.2)</td>
<td>(0.6, 8.4)</td>
<td>(8.5, 23.1)</td>
<td>(4.2, 16.2)</td>
<td>(7.0, 20.8)</td>
<td>(17.6, 35.4)</td>
<td>(0.2, 9.7)</td>
</tr>
<tr>
<td>O. venulosum</td>
<td>2.9^</td>
<td>5.0^</td>
<td>7.8^</td>
<td>10.9^</td>
<td>6.9^</td>
<td>19.8^</td>
</tr>
<tr>
<td>(0.6, 8.4)</td>
<td>(1.6, 11.2)</td>
<td>(3.4, 14.9)</td>
<td>(5.6, 18.7)</td>
<td>(2.8, 13.6)</td>
<td>(12.5, 28.9)</td>
<td>(0.0, 3.6)</td>
</tr>
<tr>
<td>McMaster WEC prevalence</td>
<td>14.6^</td>
<td>18.0^</td>
<td>75.5^</td>
<td>15.8^</td>
<td>78.4^</td>
<td>68.3^</td>
</tr>
<tr>
<td>(5.6, 29.2)</td>
<td>(0.0, 9.4)</td>
<td>(66.0, 83.5)</td>
<td>(9.3, 24.4)</td>
<td>(69.2, 86.0)</td>
<td>(58.3, 77.2)</td>
<td>(12.4, 28.6)</td>
</tr>
<tr>
<td>qPCR strongylid prevalence</td>
<td>24.5^</td>
<td>22.8^</td>
<td>83.3^</td>
<td>22.8^</td>
<td>93.1^</td>
<td>92.1^</td>
</tr>
<tr>
<td>(16.5, 34.0)</td>
<td>(15.6, 13.2)</td>
<td>(74.7, 90.0)</td>
<td>(15.0, 32.2)</td>
<td>(86.4, 97.2)</td>
<td>(85.0, 96.5)</td>
<td>(12.4, 28.6)</td>
</tr>
<tr>
<td>(\kappa) statistic ± SE</td>
<td>0.69 ± 0.14</td>
<td>0.13 ± 0.12</td>
<td>0.64 ± 0.10</td>
<td>0.88 ± 0.08</td>
<td>0.22 ± 0.08</td>
<td>0.18 ± 0.11</td>
</tr>
</tbody>
</table>

Note: Overall prevalence is the percentage of lambs that were positive for a particular parasite at any of the five samplings.

* Indicates after this sampling occasion that lambs received an anthelmintic treatment.

1 Not all samples had enough faecal material to conduct WEC (n=41 for Flocks S and L, respectively).

0 Kappa statistic; the level of agreement between the PCR assays and McMaster WEC diagnostic tests for identifying patent strongylid nematode infections ± standard error (SE).

A^ Values in columns within each individual sampling occasion that have different superscripts are significantly different between flocks (P <0.01).
Table 10.5: Arithmetic mean, adjusted strongylid faecal worm egg counts (WECs) for each lamb flock.

<table>
<thead>
<tr>
<th>Sampling occasion</th>
<th>Adjusted WEC (eggs per gram ± standard error of the mean (range))</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flock S</td>
<td>Flock L</td>
</tr>
<tr>
<td>Sampling 1</td>
<td>14 ± 6 (0 – 155)</td>
<td>1 ± 1 (0 – 77)</td>
</tr>
<tr>
<td>Sampling 2</td>
<td>164 ± 21 (0 – 1549)</td>
<td>23 ± 9 (0 – 775)</td>
</tr>
<tr>
<td>Sampling 3</td>
<td>259 ± 32 (0 – 1494)</td>
<td>194 ± 21 (0 – 1317)</td>
</tr>
<tr>
<td>Sampling 4</td>
<td>88 ± 9 (0 – 775)</td>
<td>34 ± 3 (0 – 172)</td>
</tr>
<tr>
<td>Sampling 5</td>
<td>99 ± 21 (0 – 1239)</td>
<td>46 ± 11 (0 – 697)</td>
</tr>
</tbody>
</table>

* Not all samples had enough faecal material to conduct WECs at this sampling occasion (Flock S n=57 and L n=41)

Note: P value from Mann—Whiney U non-parametric test for significance.

* Indicates after this sampling occasion that lambs received an anthelmintic treatment.
Figure 10.3: Number of strongylid species detected by qPCR per lamb at each respective sampling (A), proportions of lambs with different numbers of strongylid species detected by qPCR at each sampling occasion (B) and correlations between cycle threshold (C_q) and adjusted, log transformed WEC for animals with a single strongylid species infection (C).

10.3.2 PROTOZOAN SPECIES AND GENOTYPES

At the first sampling of the combined pregnant ewe flock (16 weeks pre-partum), the *Cryptosporidium* species detected were *C. parvum* (n=3), *C. ubiquitum* (n=6) and *C. xiaoi* (n=9). Following splitting of the combined ewe flock, the *Cryptosporidium*
species/genotypes isolated from Flock S were *C. parvum* (*n*=1), *C. ubiquitum* (*n*=4) and *C. xiaoi* (*n*=5) at 2 weeks pre-partum and *C. ubiquitum* (*n*=5) and *C. xiaoi* (*n*=4) at 4 weeks post-partum. Similarly for Flock L, *C. ubiquitum* (*n*=3) and *C. xiaoi* (*n*=4), were isolated at 2 weeks pre-partum and *C. ubiquitum* (*n*=3) and *C. xiaoi* (*n*=5) at 4 weeks post-partum.

The *Giardia* assemblages identified from the combined pregnant ewe flock samples (16 weeks pre-partum) were assemblage A (*n*=10) and assemblage E (*n*=18). After the combined ewe flock was split, the *Giardia* genotypes isolated from Flock S were assemblage A (*n*=5) and assemblage E (*n*=6) at 2 weeks pre-partum and assemblage A (*n*=6) and assemblage E (*n*=9) at 4 weeks post-partum. Genotypes isolated from Flock L were assemblage A (*n*=3) and assemblage E (*n*=5) at 2 weeks pre-partum and assemblage A (*n*=5) and assemblage E (*n*=9) at 4 weeks post-partum.

The *Cryptosporidium* and *Giardia* species/genotypes detected from each lamb flock are presented in Figure 10.4 for all samplings. The phylogenetic analyses of *Cryptosporidium* and *Giardia* species/genotypes isolated from each flock and their environment (pasture and water) are displayed in Figures 10.5 and 10.6.
Figure 10.4: *Cryptosporidium* (A and B) and *Giardia* (C and D) species/genotypes detected in lambs at each of the five sampling occasions for Flocks S and L.
Figure 10.5: Phylogenetic relationships of *Cryptosporidium* species and genotypes isolated from lambs in southern Western Australia, with some known *Cryptosporidium* species and genotypes, as inferred by Neighbour-joining analysis of Kimura’s distances calculated from pair-wise comparisons of partial (~540bp) 18S rRNA gene (A). Percentage bootstrap values (>50%) from 1000 pseudoreplicate are shown for both the Neighbour-joining (first value) and maximum likelihood (second value) analyses. ns = node with bootstrap value <50%.
Figure 10.6: Phylogenetic relationships of *Giardia duodenalis* assemblages isolated from lambs in southern Western Australia, with some known assemblages, as inferred by Neighbour-joining analysis of Kimura's distances calculated from pair-wise comparisons of partial (~480bp) *glutamate dehydrogenase* (B). Percentage bootstrap values (>50%) from 1000 pseudoreplicate are shown for both the Neighbour-joining (first value) and maximum likelihood (second value) analyses. ns = node with bootstrap value <50%.
10.3.3 LEVELS OF AGREEMENT BETWEEN qPCR AND McMaster WEC RESULTS

For detection of patent strongylid infections, the overall level agreement ($\kappa$ value) between qPCR and WEC results was $0.77 \pm 0.02$ for both flock results combined. The levels of agreement between the McMaster WEC and the PCR in identifying patent strongylid infections ($\kappa$ statistic) are shown in Table 10.4 for each flock.

A total of 17/1015 samples (1.7%) were McMaster WEC flotation positive (50 epg) and qPCR negative. Following spiking of these sample extracts with 1 $\mu$L purified DNA from each of the five strongylid species, the spiked DNA mixture was screened by the qPCRs to test for inhibition. All qPCRs amplified in accordance with positive controls, indicating that these samples did not contain co-purified PCR inhibitors.

A total of 84/1015 samples (8.3%) that were McMaster WEC negative and qPCR positive were re-screened, with the PCR products sequenced. The sequenced products *T. circumcincta* ($n = 50$), *T. colubriformis* ($n = 23$), *H. contortus* ($n = 7$), *C. ovina* ($n = 3$) and *O. venulosum* ($n = 1$) were 100% identical with GenBank reference sequences, confirming the initial qPCR results.

10.3.4 INTERNAL PARASITES RECOVERED FROM THE ENVIRONMENT OF EACH FLOCK

The pasture larval count results are shown in Figure 10.7 and Table 10.6. The total larvae/kg of pasture dry weight was significantly different between Flock S and L on each of the final six pasture sampling occasions ($P<0.05$). *Trichostrongylus* spp. and *T. circumcincta* were the strongylid species most commonly detected by the qPCRs and also
in the pasture larval counts. *Nematodirus* spp. was isolated only from Paddock S at low levels on the July sampling (Figure 10.7).

The minimum amount of genomic DNA required for successful qPCR amplification of the *T. colubriformis* and *T. circumcincta* spiked pasture samples were 0.1 pg and 1 pg, respectively. The qPCR results using genomic DNA extracted directly from material obtained by the modified strongylid larvae recovery procedure are shown in Table 10.6. The sequenced products of *T. circumcincta* (n=71), *Trichostrongylus colubriformis* (n=94), *H. contortus* (n=19) and *C. ovina* (n=7) were aligned with reference sequences on GenBank and were 100% identical.

For Paddock S, *T. circumcincta* was detected by qPCR in 4/10 of the pasture sub-samples (July sampling) and *H. contortus* was identified by qPCR in 2/10 of the pasture sub-samples (September sampling), despite these species not being observed in the pasture larvae counts at the respective samplings (Table 10.6). Cryptosporidia (*C. ubiquitum* n=2 and *C. xiaoi* n=1) were identified in 3/10 of the pasture sub-samples and *Giardia* (assemblage E n=2) were identified in 2/10 pasture sub-samples at the August sampling. Cryptosporidia (*C. ubiquitum*) were identified in 1/10 of the pasture sub-samples and *Giardia* (assemblage A n=2 and assemblage E n=1) were identified in 3/10 pasture sub-samples at the September sampling. *Giardia* (assemblage A [n=1] and assemblage E [n=3]) in 4/10 pasture sub-samples at the August sampling for Paddock L. Cryptosporidia (*C. xiaoi* [n=2]) were identified in 2/10 pasture sub-samples at the September sampling.
The dam water sourced by lambs from Flock S (Figure 10.8) tested positive for *Giardia* assemblage E at the second and third sampling occasions, but not the first. Furthermore, the same dam water tested positive for *C. ubiquitum*, but only at the second sampling. The trough water supplied to Flock L lambs (Figure 10.9) was sourced from a dam by an underground piping system and never tested positive for *Cryptosporidium* or *Giardia* at any of the three water sampling occasions.
Figure 10.7: Strongylid species pasture larval counts (larvae/kg pasture DM) for each paddock (S and L).
Figure 10.8: Dam water sourced by Flock S grazing Paddock S.
Figure 10.9: Trough water sourced by Flock L grazing Paddock L. Water was supplied to troughs from a dam by an underground piping system.
Table 10.6: Pasture larval count results and qPCR results from modified strongylid larvae recovery procedure.

<table>
<thead>
<tr>
<th>Sampling occasion</th>
<th>Paddock S</th>
<th>Paddock L</th>
<th>Paddock S</th>
<th>Paddock L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pasture larvae count</td>
<td>Real time PCR ‘modified strongylid larvae recovery procedure’</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pasture dry weight (g)</td>
<td>Larval species/ kg pasture dry weight (r)</td>
<td>Total larvae/kg pasture dry weight (r)</td>
<td>Pasture dry weight (g)</td>
</tr>
<tr>
<td>March 22th</td>
<td>105.2</td>
<td>0</td>
<td>107.1</td>
<td>0</td>
</tr>
<tr>
<td>April 23rd</td>
<td>88.1</td>
<td>0</td>
<td>101.4</td>
<td>0</td>
</tr>
<tr>
<td>May 25th</td>
<td>69.8</td>
<td>0</td>
<td>85.2</td>
<td>0</td>
</tr>
<tr>
<td>June 15th</td>
<td>96.8</td>
<td>Trich =234</td>
<td>94.9</td>
<td>82.2</td>
</tr>
<tr>
<td>July 6th</td>
<td>105.0</td>
<td>Trich =1297</td>
<td>110.6</td>
<td>0</td>
</tr>
<tr>
<td>August 23rd</td>
<td>111.7</td>
<td>Trich =3501</td>
<td>70.6</td>
<td>Trich =143,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Nem =162</td>
<td>Nem =75</td>
</tr>
<tr>
<td>September 19th</td>
<td>88.5</td>
<td>Trich =4106</td>
<td>71.3</td>
<td>Trich =967</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tel =1497</td>
<td>Tel =623</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chab =120</td>
<td></td>
</tr>
<tr>
<td>October 6th</td>
<td>78.0</td>
<td>Trich =2405</td>
<td>65.2</td>
<td>Trich =2307</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tel =1703</td>
<td>Tel =1070</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>Haem =306</td>
<td>Haem =131</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chab =127</td>
<td></td>
</tr>
<tr>
<td>November 15th</td>
<td>108.9</td>
<td>Trich =6014</td>
<td>81.1</td>
<td>Trich =1257</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tel =614</td>
<td>Tel =1816</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Haem =325</td>
<td>Haem =253</td>
</tr>
</tbody>
</table>

Note: T. circumcincta = Tel, Trichostrongylus spp. = Trich, H. contortus = Haem, C. ovina = Chab, Oesophagostomum spp. = Oesoph and Nematodirus = Nem.

Strongylid species were detected from the ten separate DNA extractions from material caught during the modified strongylid larvae recovery procedure. Nematodirus was not screened for by PCR.
10.3.5 LEVELS OF AGREEMENT BETWEEN qPCR AND PASTURE LARVAE COUNT RESULTS

When assessing results for the known pasture samples (spiked with known numbers of \textit{T. circumcincta} or \textit{T. colubriformis} L\textsubscript{3}) in comparison with the qPCR results for strongylid species detection, 12/120 (10.0\%) of the sub-samples returned a negative qPCR result for the detection of strongylid species.

The overall level of agreement between the field pasture larvae counts and qPCR results for detecting the presence of strongylid larval species on pasture was 0.67 \(\pm\) 0.04 (\(P<0.001\)). A total of 46/310 (14.8\%) sub-samples returned a negative qPCR result for the detection of strongylid species when the pasture larvae count returned a positive result (larvae detected/kg of dry matter).

The average and range of \(C_q\) values for each strongylid species detected from the pasture sub-samples are displayed in Table 10.6. The correlations between qPCR \(C_q\) and log-transformed pasture larvae counts (both field and spiked samples) are illustrated in Figure 10.10 for \textit{Trichostrongylus} spp. and \textit{T. circumcincta}. 
Figure 10.10: Comparison of cycle threshold ($C_q$) values in qPCR with pasture larval count numbers (larvae/kg of dry matter) for *Trichostrongylus* spp. (A) or *T. circumcincta* (B). PCR amplification was from genomic DNA extracted from material caught following the modified strongyelid larvae recovery procedure for pastures spiked with known quantities of strongyelid species larvae (closed diamond), or pasture samples collected from field Paddock S and L (closed square).
10.3.6 BETWEEN FLOCK ANALYSES – PRODUCTION PERFORMANCES AND FAECAL ATTRIBUTES IN LAMBS WITH DIFFERENT NATURAL LARVAL CHALLENGE

10.3.6.1 Hot carcase weight and dressing percentage

There were no significant differences between the average hot carcase weight and fat scores of each flock. The average dressing percentage of Flock S was 1.52% lower than that of Flock L (P=0.038) (Table 10.7).

10.3.6.2 Growth rate, live weight and BCS

There were no significant differences between the average live weights of each flock at the five sampling occasions, except on the final sampling occasion when Flock S had greater live weight than Flock L (P=0.048) (Table 10.7). There were also no significant differences between the average growth rate (both grams/day gained and percentage of previous sampling live weight gained) of each flock at the five sampling occasions, except between the fourth and final samplings when Flock S had greater growth rates than Flock L (P=0.033) (Table 10.7). The average BCSs for each lamb flock were not significantly different to each other at the four sampling occasions where BCS was recorded (Table 10.7).
Table 10.7: Mean production attributes (hot carcase weight, dressing percentage, live weight, growth rate and body condition score) for each lamb flock.

<table>
<thead>
<tr>
<th>Production attribute</th>
<th>Flock S</th>
<th>Flock L</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hot carcase weight (kg)</td>
<td>17.75 ± 0.28</td>
<td>17.28 ± 0.28</td>
<td>0.235</td>
</tr>
<tr>
<td>Dressing percentage</td>
<td>42.45 ± 0.22</td>
<td>43.97 ± 0.21</td>
<td>0.038</td>
</tr>
<tr>
<td>GR knife fat depth (mm)</td>
<td>1.80 ± 0.06</td>
<td>1.83 ± 0.06</td>
<td>0.735</td>
</tr>
<tr>
<td>Live weight (kg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sampling 1</td>
<td>14.12 ± 0.40</td>
<td>13.63 ± 0.29</td>
<td>0.312</td>
</tr>
<tr>
<td>Sampling 2</td>
<td>26.97 ± 0.48</td>
<td>25.87 ± 0.49</td>
<td>0.114</td>
</tr>
<tr>
<td>Sampling 3</td>
<td>36.78 ± 0.40</td>
<td>36.30 ± 0.29</td>
<td>0.545</td>
</tr>
<tr>
<td>Sampling 4</td>
<td>37.35 ± 0.52</td>
<td>36.87 ± 0.52</td>
<td>0.231</td>
</tr>
<tr>
<td>Sampling 5</td>
<td>41.33 ± 0.56</td>
<td>39.75 ± 0.57</td>
<td>0.048</td>
</tr>
<tr>
<td>Growth rate (grams gained/day)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sampling 1 to 2</td>
<td>305.8 ± 7.6</td>
<td>291.3 ± 7.6</td>
<td>0.181</td>
</tr>
<tr>
<td>Sampling 2 to 3</td>
<td>233.7 ± 8.4</td>
<td>248.4 ± 8.5</td>
<td>0.221</td>
</tr>
<tr>
<td>Sampling 3 to 4</td>
<td>17.1 ± 9.5</td>
<td>9.28 ± 9.6</td>
<td>0.379</td>
</tr>
<tr>
<td>Sampling 4 to 5</td>
<td>128.1 ± 7.3</td>
<td>105.8 ± 7.3</td>
<td>0.033</td>
</tr>
<tr>
<td>Growth rate (% of previous sampling live weight gained)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sampling 1 to 2</td>
<td>98.73 ± 3.44</td>
<td>94.29 ± 3.45</td>
<td>0.365</td>
</tr>
<tr>
<td>Sampling 2 to 3</td>
<td>38.39 ± 2.11</td>
<td>41.86 ± 1.61</td>
<td>0.195</td>
</tr>
<tr>
<td>Sampling 3 to 4</td>
<td>2.40 ± 0.89</td>
<td>1.82 ± 0.90</td>
<td>0.210</td>
</tr>
<tr>
<td>Sampling 4 to 5</td>
<td>9.54 ± 0.54</td>
<td>8.05 ± 0.55</td>
<td>0.054</td>
</tr>
<tr>
<td>Body condition score</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sampling 2</td>
<td>2.39 ± 0.05</td>
<td>2.31 ± 0.05</td>
<td>0.227</td>
</tr>
<tr>
<td>Sampling 3</td>
<td>2.90 ± 0.05</td>
<td>2.82 ± 0.05</td>
<td>0.241</td>
</tr>
<tr>
<td>Sampling 4</td>
<td>2.91 ± 0.04</td>
<td>2.91 ± 0.03</td>
<td>0.954</td>
</tr>
<tr>
<td>Sampling 5</td>
<td>3.19 ± 0.05</td>
<td>3.11 ± 0.05</td>
<td>0.233</td>
</tr>
</tbody>
</table>

Note: Production attribute mean ± standard error of the mean.

Body condition score (BCS) was not recorded at the first sampling.

P value from ANOVA test for significance.

10.3.6.3 Faecal consistency score, FDM% and breech fleece faecal soiling score

At both the first (P=0.029) and second (P=0.002) samplings, Flock S had FCSs ~0.3 higher than Flock L (Table 10.8). At the second sampling, Flock S had FDM%~ 3.5% lower than Flock L and breech fleece faecal soiling scores ~0.4 higher than Flock L (P=0.001). A trend was observed at the fourth sampling, where Flock S had FDM%~ 1.0%
lower than Flock L (P=0.092) and breech fleece faecal soiling scores ~0.15 higher than Flock L (P=0.085) (Table 10.8).

**Table 10.8:** Mean faecal attributes (faecal consistency score [FCS] and faecal dry matter percentage [FDM%]) for each lamb flock.

<table>
<thead>
<tr>
<th>Faecal attribute</th>
<th>Flock S</th>
<th>Flock L</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Faecal consistency score (FCS)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sampling 1</td>
<td>1.62 ± 0.09</td>
<td>1.37 ± 0.08</td>
<td>0.029</td>
</tr>
<tr>
<td>Sampling 2</td>
<td>3.08 ± 0.07</td>
<td>2.77 ± 0.07</td>
<td>0.002</td>
</tr>
<tr>
<td>Sampling 3</td>
<td>3.03 ± 0.06</td>
<td>2.98 ± 0.05</td>
<td>0.551</td>
</tr>
<tr>
<td>Sampling 4</td>
<td>2.69 ± 0.09</td>
<td>2.59 ± 0.09</td>
<td>0.423</td>
</tr>
<tr>
<td>Sampling 5</td>
<td>3.02 ± 0.06</td>
<td>2.91 ± 0.07</td>
<td>0.234</td>
</tr>
<tr>
<td>Faecal dry matter percentage (FDM%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sampling 1</td>
<td>34.89 ± 0.78</td>
<td>36.66 ± 0.97</td>
<td>0.155</td>
</tr>
<tr>
<td>Sampling 2</td>
<td>18.51 ± 0.45</td>
<td>22.04 ± 0.45</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sampling 3</td>
<td>16.84 ± 0.34</td>
<td>16.87 ± 0.28</td>
<td>0.947</td>
</tr>
<tr>
<td>Sampling 4</td>
<td>24.12 ± 0.49</td>
<td>25.17 ± 0.48</td>
<td>0.092</td>
</tr>
<tr>
<td>Sampling 5</td>
<td>23.59 ± 0.33</td>
<td>25.19 ± 0.32</td>
<td>0.001</td>
</tr>
<tr>
<td>Breech fleece faecal soiling</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sampling 2</td>
<td>1.64 ± 0.09</td>
<td>1.22 ± 0.08</td>
<td>0.001</td>
</tr>
<tr>
<td>Sampling 3</td>
<td>2.31 ± 0.08</td>
<td>2.01 ± 0.08</td>
<td>0.038</td>
</tr>
<tr>
<td>Sampling 4</td>
<td>1.77 ± 0.07</td>
<td>1.62 ± 0.06</td>
<td>0.085</td>
</tr>
<tr>
<td>Sampling 5</td>
<td>1.96 ± 0.08</td>
<td>1.85 ± 0.08</td>
<td>0.313</td>
</tr>
</tbody>
</table>

Note: Production attribute mean ± standard error of the mean.

P value from ANOVA test for significance.

10.3.7 WITHIN FLOCK ANALYSES – RELATIONSHIP BETWEEN PARASITES AND COMPARING CARCASE PRODUCTION PERFORMANCES

Detection of *Giardia* on at least one sampling occasion was associated with 1.25kg (7.0%) lower HCWs for Flock S (P=0.034) and 1.02kg (5.9%) lower HCWs for Flock L (P=0.048), compared to lambs which never tested positive for *Giardia* (Table 10.9). Lambs *Cryptosporidium*-positive on one or more samplings had 1.14% (P=0.050) lower and 1.77%
lower dressing percentage for Flocks S and L, respectively. Lambs positive for *Giardia* on one or more samplings had 1.42% (P=0.044) lower dressing percentage for Flocks L only (Table 10.9). Lambs positive for *Giardia* on two or more occasions had lower dressing percentages by 1.61% (P=0.032) and 1.95% (P=0.030) when compared to lambs positive for *Giardia* once or never at all Flocks S and L, respectively.

Fat scores of *Cryptosporidium*-positive lambs (P=0.035) and *Giardia*-positive lambs (P=0.033) were 0.25 lower when compared to negative lambs in Flock S only (Table 10.9).

General linear regression analysis revealed adjusted, log-transformed WEC covariate was not correlated with any carcase attributes for either flock (Table 10.9).
Table 10.9: Associations/correlations between Cryptosporidium and Giardia detection, along with adjusted, log-transformed WEC with carcase attributes for each flock.

<table>
<thead>
<tr>
<th>Carcase attribute</th>
<th>Protozoa-negative</th>
<th>Protozoa-positive</th>
<th>P value</th>
<th>WEC linear P value</th>
<th>WEC r^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hot carcase weight (HCW)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryptosporidium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flock S</td>
<td>18.09 ± 0.46</td>
<td>17.51 ± 0.39</td>
<td>0.338</td>
<td>0.525</td>
<td>0.011</td>
</tr>
<tr>
<td>Flock L</td>
<td>17.37 ± 0.36</td>
<td>17.17 ± 0.39</td>
<td>0.703</td>
<td>0.202</td>
<td>0.041</td>
</tr>
<tr>
<td>Giardia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flock S</td>
<td>18.57 ± 0.43</td>
<td>17.32 ± 0.41</td>
<td>0.034</td>
<td>0.525</td>
<td>0.011</td>
</tr>
<tr>
<td>Flock L</td>
<td>17.72 ± 0.42</td>
<td>16.70 ± 0.44</td>
<td>0.048</td>
<td>0.202</td>
<td>0.041</td>
</tr>
<tr>
<td>Dressing percentage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryptosporidium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flock S</td>
<td>43.52 ± 0.44</td>
<td>42.38 ± 0.37</td>
<td>0.050</td>
<td>0.298</td>
<td>0.031</td>
</tr>
<tr>
<td>Flock L</td>
<td>44.29 ± 0.34</td>
<td>42.52 ± 0.37</td>
<td>0.001</td>
<td>0.752</td>
<td>0.025</td>
</tr>
<tr>
<td>Giardia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flock S</td>
<td>42.96 ± 0.42</td>
<td>42.76 ± 0.40</td>
<td>0.723</td>
<td>0.298</td>
<td>0.031</td>
</tr>
<tr>
<td>Flock L</td>
<td>43.98 ± 0.43</td>
<td>42.53 ± 0.44</td>
<td>0.044</td>
<td>0.752</td>
<td>0.025</td>
</tr>
<tr>
<td>Fat score</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryptosporidium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flock S</td>
<td>1.95 ± 0.09</td>
<td>1.70 ± 0.07</td>
<td>0.035</td>
<td>0.469</td>
<td>0.014</td>
</tr>
<tr>
<td>Flock L</td>
<td>1.89 ± 0.08</td>
<td>1.77 ± 0.08</td>
<td>0.279</td>
<td>0.659</td>
<td>0.024</td>
</tr>
<tr>
<td>Giardia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flock S</td>
<td>1.94 ± 0.09</td>
<td>1.69 ± 0.07</td>
<td>0.033</td>
<td>0.469</td>
<td>0.014</td>
</tr>
<tr>
<td>Flock L</td>
<td>1.88 ± 0.07</td>
<td>1.75 ± 0.09</td>
<td>0.314</td>
<td>0.659</td>
<td>0.024</td>
</tr>
</tbody>
</table>

10.4 DISCUSSION

The present study compared differences in flock production performances between two flocks of lambs grazing pastures with different levels of strongylid larval contamination. Larval contamination of pastures was assessed using both a conventional method (Martin et al., 1990; Animal Health Laboratories, 2005b) and an innovative, molecular modified technique. Differences in production attributes (growth rate) and faecal attributes were observed in 3/5 samplings, along with differences in the strongylid species prevalences. Lambs exposed to a higher larval challenge had lower dressing percentages, but there was no effect observed on HCW. Within flock general linear analyses found that Cryptosporidium and Giardia were negatively associated with carcase attributes for both
flocks and adjusted, log-transformed WEC was not correlated with any carcase attributes (Table 10.9).

10.4.1 STRONGYLIDS AND LAMB PRODUCTION PERFORMANCES

Lambs in Flock S that were exposed to a higher strongylid larval challenge, had heavier live weights at the final sampling and greater growth rates between the last 2 samplings when compared to Flock L (Table 10.7). Notably, lambs exposed to a lower level of larval challenge (Flock L) had greater dressing percentages (Table 10.7). However, there was no significant difference in the HCWs between each flock. The differences in live weights and growth rates observed between flocks could be attributed to different patterns of exposure to L₃, specifically constant high exposure in Flock S, versus incremental exposure starting at low levels in Flock L. The acquisition of immunity to strongylid parasites is determined by the level and duration of larval challenge (Dobson et al., 1990b, d; Barnes and Dobson, 1993). It is possible that lambs exposed to higher pasture larval levels earlier in this study (Flock S), would have had initiation of acquired immune responses and an associated depression of appetite earlier in this present study, when compared with lambs in Flock L (Greer, 2008; Greer et al., 2008). This hypothesis is supported by the finding that lambs in Flock L had greater growth rates between the first and second samplings. In contrast, lambs in Flock L were not exposed to moderate levels of strongylid L₃ until later in the study (fourth sampling). Therefore the differences in final live weight and growth rates from the fourth to final sampling is likely a result of Flock L lambs having later initiation with their development of immunity to strongylids and suppressed appetite later in the study.
Many factors impact lamb production performances and although parasitism is one important factor, nutrition (quality and quantity of feed), genetics, time of weaning, sex and the ratio of single to twin born lambs, all impact very strongly on overall flock performance (Makarechian et al., 1978; Arnold and Meyer, 1988; Coop and Sykes, 2002; Kahn et al., 2003; Kenyon et al., 2004; Houdijk, 2008; Hatcher et al., 2009). Such factors are also critical for lambs in maintaining good body condition (van Burgel et al., 2011). There was no evidence that differences in feed quantity (FOO) or quality (digestibility and protein) were sufficiently different between the paddocks to explain the differences observed. Lamb birth type (single or twin) was not recorded in the present study because sheep were managed under extensive conditions that made supervision of lambing and recording of birth type impractical.

Although there were differences in live weight between the two flocks at the final sampling, there was no significant difference in the HCWs between each flock. Lambs exposed to lower levels of larval challenge (Flock L) had greater dressing percentages when compared to those of Flock S (Table 10.7). Other studies have reported heavier intestinal weights relative to the overall live weight and reduced dressing percentage (carcase weight as a proportion of live weight) of strongylid infected sheep (Liu et al., 2003; Jacobson et al., 2009c), but the relationship between level of larval challenge and intestinal weight has not been well described.

Strongylid L₃ challenge has been reported to increase the risk of sheep producing faeces with higher moisture content (Larsen et al., 1994; Larsen and Anderson, 2000; Williams et al., 2010a; Williams et al., 2010b). Lambs exposed to higher larval challenge levels (Flock S) had higher faecal scores (more loosely formed faeces) than lambs in Flock
L at the first two samplings (Table 10.8) and increased faecal moisture at the first sampling, when the larval contamination on Paddock L was at its lowest (Figure 10.7). The differences in faecal consistency were reflected in the breech soiling scores, whereby lambs exposed to a higher level larval challenge (Flock S), had more extensive breech fleece faecal soiling at the second and third samplings (P<0.05) compared to lambs in Flock L (Table 10.8). This is consistent with previous studies which showed that increasing L₃ challenge increased FCSs and increased breech fleece faecal soiling scores (Larsen et al., 1994; Larsen and Anderson, 2000).

Pregnant ewes were introduced onto Paddock L after they were treated with an effective anthelmintic (monepantel). Despite this, strongylid larvae contamination on this pasture was observed, but at a much slower rate and at a lower level, in comparison to the challenge recorded on Paddock S. Potentially, the source of pasture contamination may have been due to hypobiotic larvae that may have survived treatment, as *H. contortus* and *T. circumcincta* were detected in lambs and on pastures from Paddock L, with both these strongylid species reported as having the capability of arrested larval development (Gibbs, 1986).

### 10.4.2 Protozoa and Lamb Production Performances

*Cryptosporidium* and *Giardia* were detected in both ewes and lambs from each flock, while *Cryptosporidium* and *Giardia* were also identified in some of the genomic DNA extracted from pasture sub-samples from each paddock, with different species/assemblages discovered at a single sampling occasion. Within flock general linear analyses found that *Cryptosporidium* and *Giardia* were negatively associated with flock...
carcase attributes for both flocks and adjusted, log-transformed WEC was not correlated with any carcase attributes (Table 10.9).

General linear regression analyses within each flock identified that reduced HCWs were associated with *Giardia* detection (Table 10.9). For both flocks, average HCWs were 6–7% lighter for lambs that tested positive for *Giardia* on at least one sampling occasion. Lambs positive for *Cryptosporidium* had reduced dressing percentages in both flocks, while *Giardia*-positive lambs had reduced dressing percentages in only one flock (Flock L). These carcase attributes are key profit drivers for meat lamb enterprises and this is the second report of an association between protozoa species and negative impacts on carcase attributes for extensively grazed lambs (Chapter Nine). In addition there is another report of reduced HCWs in barn-raised lambs surgically infected with *Giardia* trophozoites (Olson et al., 1995). Therefore further research is warranted to assess whether similar effects are observed in other livestock and whether quantification of these protozoan infections reveals relationships between intensity of infection and productivity.

The mechanism by which protozoa infection may negatively impact carcase productivity is not well described. Protozoa have been reported to cause inflammation of the gastrointestinal tract (Scott et al., 2002; Buret et al., 2003). Inflamed gastrointestinal organs could contribute towards reduced dressing percentage. There are many other factors which influence dressing percentage (time off feed, wool length, breed, time of weaning and gender), all of which were kept consistent for each of the two flocks in this study.

Despite the detection of *Cryptosporidium* and *Giardia* associated with reduced fat scores for Flock S, the likelihood that this finding was due to parasitism is unlikely. A
greater range in fat score units (1.0) compared to the more precise measurement of GR fat depth units (0.1), potentially is the major cause of the significant differences observed.

10.4.3 PROTOZOA PREVALENCE IN SHEEP AND DAM WATER

*Cryptosporidium* and *Giardia* were identified in pregnant ewes and their lambs from both flocks. Each farm had over 58% of the identified lambs test positive for *Cryptosporidium* on at least one of the five samplings and over 46% test positive for *Giardia*. There were higher sampling prevalences of these protozoa in the ewes (Table 10.3), when compared to the lambs from each flock (Table 10.4). The sampling prevalence range for the lambs was notably lower when compared to previous studies in Australia (Chapter Seven and Eight).

The dam water sourced by lambs from Flock S (Figure 10.8) tested positive for *Giardia* assemblage E at both the second and third sampling occasions, but not the first. Furthermore, the same dam water tested positive for *C. ubiquitum*, but only at the second sampling. The trough water supplied to Flock L lambs was sourced from a dam by an underground piping system (Figure 10.9) and never tested positive for *Cryptosporidium* or *Giardia* on any of the three water sampling occasions. This is the second report of protozoa detection in livestock dam water in Australia (Chapter Seven), with some of the isolates from dam water identical to isolates obtained from lambs. Although zoonotic *C. parvum* was isolated from both flocks (Figures 10.4 and 10.5), the *Cryptosporidium* public health risk was low due to *C. xiaoi* and *C. ubiquitum* being the most frequently detected species. *Giardia* zoonotic assemblage A was routinely detected in both flocks, nearly as often as assemblage E and as a result both flocks were potential sources of zoonotic *Giardia*. Further investigation is warranted to examine the contamination risk that different water
sources pose for both livestock and human drinking water (Hooda et al., 2000; Dixon et al., 2011; Ng et al., 2011b).

10.4.4 MODIFIED MOLECULAR TECHNIQUE FOR RECOVERING STRONGYLID LARVAE FROM PASTURES

An innovative, molecular qPCR diagnostic approach was utilised to recover, identify and quantify those strongylid larvae species that exist on pasture and results were compared to the traditional pasture larval count procedure. There was a moderate level of agreement (67%) between pasture larval counts and qPCR results. Moreover, there was a strong, negative correlation between qPCR $C_q$ values ($Trichostrongylus$ spp. and $T. circumcincta$) and log-transformed pasture larval counts from known larvae spiked samples and field samples with unknown levels of contamination ($r^2 > 0.91$).

The moderate overall level of agreement observed between pasture larvae counts and qPCR results was due to ~15% of pasture sub-samples returning negative qPCR results, when pasture larvae counts confirmed that strongylid larvae were present on pasture (Table 10.4). This indicates that the procedure employed to recover larvae from pastures for DNA extraction requires further development to improve the recovery rate of strongylid larvae. At present, genomic DNA extractions would be necessary on a total of 10 sub-samples on the material recovered utilising the modified pasture larvae recovery procedure. Despite molecular diagnostic techniques requiring less labour and possessing greater diagnostic sensitivity, the cost-benefits of these techniques are an important factor in determining their uptake by diagnostic laboratories and also if livestock enterprises are willing to pay for them (Hunt, 2011).
While there is potential for using molecular diagnostic techniques for measuring larvae from pastures, there is little encouragement in the scientific literature that pasture larvae counts are widely utilised as a diagnostic/predictive tool to aid grazing management (Gettinby et al., 1985; Martin et al., 1990; Couvillion, 1993; Fine et al., 1993; Watson, 2007). One of the limitations of pasture larval counts is the time involved, specifically the traditional method is cumbersome and time consuming (7–10 days to conduct), creating a time lapse between pasture collection and larvae recovery and reducing the proportion of larvae recovered (Gettinby et al., 1985; Fine et al., 1993). Results using this modified molecular technique were derived within 1½ days of pasture collection, so the modified recovery method and molecular method does offer advantages from a time perspective. By screening genomic DNA with qPCR assays, it reveals an opportunity for high sample throughput and automation, with pasture collection from the paddock the only remaining unwieldy task.

Furthermore, there are several different variations in the methodology used for pasture larvae counts, with some suggestions that low strongylid larvae challenges are often underestimated (Martin et al., 1990; Couvillion, 1993). The use of a ‘tracer’ sheep method has the advantage whereby selected animals mimic similar grazing patterns of the resident flock. However, high larval challenges have been reported to be underestimated using this method (Dobson et al., 1990b; Martin et al., 1990). This can be exacerbated by using animals which have a different immunity status to the target animals. These issues are the main reasons why many diagnostic laboratories choose not to perform this procedure. The qPCRs utilised in this study were capable of detecting between 0.1 and 1.0pg of genomic DNA from the *T. colubriformis* and *T. circumcincta* spiked pasture
samples respectively, which indicates greater sensitivity than in a previous study using conventional PCR assays (Chapter Six).

Genomic DNA extracts can also be screened for a multiple variety of pathogens (parasites, bacteria and viruses), with protozoa parasites detected in pasture recovered from both paddocks. *Cryptosporidium* and *Giardia* (oo)cysts have been reported to attach to soil and debris vegetation particles (Medema *et al*., 1998; Medema and Schijven, 2001; Smith and Nichols, 2010) and can survive in favourable environments for up to six months (Robertson *et al*., 1992; Olson *et al*., 1999). It is possible that if soil, vegetation or faecal particles with protozoa (oo)cysts attached, were caught by the sieves in the modified pasture larvae recovery procedure, these (oo)cysts could be detected by PCR following extraction of genomic DNA from the sub-sample.

### 10.4.5 MOLECULAR TECHNIQUE FOR IDENTIFYING STRONGYLID INFECTIONS IN SHEEP

Previous conventional PCR studies had reported an overall high (>93%) level of agreement between WEC and PCR results in detecting patent strongylid infections in faecal samples (Chapters Five and Six). However in this present study where qPCR assays were used, the overall level of agreement was moderate (77%) and had a large range across the five samplings of each flock 13–100% (Table 10.4). There was a weak correlation ($r^2=0.25$) between qPCR $C_q$ values and log-transformed, adjusted WEC (Figure 10.3 [C]), in addition to higher strongylid qPCR prevalence when compared to strongylid WEC prevalence (Table 10.4). This suggests that false positives were identified by qPCR, possibly due to non-patent strongylid DNA sources (larval tissues passing through the sheep or rupture larval DNA from hypobiotic nodules) present in genomic DNA extracts.
Alternatively, qPCR detects egg counts below the limit of detection for the conventional test (50 epg), as shown by the sensitivity analysis undertaken for pasture sampling.

10.5 CONCLUSION

This study showed that a lamb flock grazing a paddock with a higher natural strongylid larvae challenge, had significantly greater FCSs, higher breech fleece faecal soiling scores and lower dressing percentages, when compared to a lamb flock grazing a paddock with a lower strongylid pasture challenge. This suggests that gastrointestinal organs from Flock S lambs potentially had greater percentage weights due to a greater strongylid challenge. Moreover, along with significant differences in larval challenges observed between paddocks, protozoan parasites had similar impacts on the carcase productivity of each flock, consistent with results observed in Chapter Nine. Polymerase chain reaction detection of Giardia was associated with reduced HCWs in both flocks and PCR detection of Cryptosporidium was associated with reduced dressing percentages in both flocks, similar to findings reported in Chapter Nine. An innovative method to recover, identify and quantify strongylid species on pasture using qPCR was developed and tested. The technique had a moderate level of agreement with pasture larval count results and a strong, negative correlation was observed between qPCR Cq value and log-transformed pasture larvae counts. However, extensive optimisation and validation are necessary before such a molecular method can be utilised routinely by diagnostic laboratories, along with an assessment as to whether in-field tests can be designed. The detection of protozoa in ewes, lambs, flock water and recovered pasture material indicates these protozoa are present in sheep enterprises, with further work necessary to bridge the current knowledge gaps. There is potential for livestock enterprises to utilise the molecular techniques
employed in this study for early detection of internal parasites in their environment, to enhance their management and increase downstream profitability.
CHAPTER 11: GENERAL DISCUSSION AND CONCLUSIONS

11.1 DISCUSSION

The focus of this thesis was to examine the epidemiology and potential pathogenesis of protozoan and strongylid parasites in extensively grazing lambs. Molecular diagnostic techniques to identify strongylid parasites were firstly compared to traditional microscopic examination and floatation/sedimentation methods, to assess their level of agreement and the reliability of results. Secondly, statistical analyses were performed to determine whether significant associations, correlations or risks existed between lamb phenotypic production attributes and the presence of internal parasites (protozoa and strongylid worms). These molecular tools provided the discriminatory capability; to perform phylogenetic analyses to assess the genetic variation between parasite isolates, to evaluate changing internal parasite species demographics, to assess the zoonotic potential of any parasite species detected and to highlight potential routes of contamination (pasture, water or wildlife).

11.1.1 ASSOCIATIONS BETWEEN PHENOTYPIC PRODUCTION PERFORMANCE TRAITS AND THE DETECTION OF PARASITES USING MOLECULAR TECHNIQUES

A consistent finding observed in both Chapters Nine and Ten was that lambs from four flocks on three farms that were identified as protozoa-positive, had reduced carcase HCWs and dressing percentages. In Chapter Nine, Cryptosporidium-positive lambs...
(positive for Cryptosporidium on at least one of the five sampling occasions) from both farms had reduced HCWs and dressing percentages, when compared to lambs that never tested positive for Cryptosporidium. Reduced HCWs and dressing percentages were also observed when comparing Giardia-positive and Giardia-negative lambs, but only in one of the two flocks examined. In Chapter Ten, Giardia-positive lambs from both flocks also had lighter HCWs, when compared to lambs never Giardia-positive. Furthermore, Giardia-positive lambs had reduced dressing percentages when compared to negative lambs, but this was only observed in one of the flocks. In both Chapters Nine and Ten, lambs that were Cryptosporidium-positive on two or more sampling occasions had lighter HCWs when compared to lambs Cryptosporidium-positive on only one sampling, or not at all. Despite different natural strongylid pasture larvae challenges between the two flocks (S and L) in Chapter Ten, the impact that protozoan parasites had on lamb carcase productivity was similar in both flocks. The consistent findings of reduced HCWs and dressing percentages associated with protozoa detection suggests protozoa are potentially important pathogens that are capable of limiting lamb carcase production and hence profitability. Detection of protozoa in Chapter Ten was associated with reduced fat scores, however this was not consistent with the findings of Chapter Nine. A more precise measure for carcase fat (GR fat depth) was used in Chapter Nine. A greater range in fat score unites (1.0) compared to GR fat depth units (0.1) potentially attributed to the significant differences observed, rather than protozoan parasite pathogenesis.

Administering chemotherapeutic treatments which target these protozoa to livestock is a questionable management practice. Whether such treatments are cost-beneficial for sheep enterprises is debatable (Thompson et al., 2008; De Waele et al., 2010). However, other concerns warranting consideration are that administering such treatments are often
time consuming (Edlind et al., 1990; Viu et al., 2000), have variable efficacy (Edlind et al., 1990; Castro-Hermida et al., 2001; Giadinis et al., 2008; De Waele et al., 2010) and are commonly regarded as an impractical option for grazing livestock. This is because of the high risk re-infection from their environment (Thompson et al., 2008). Potential management and control practices sheep enterprises can employ to limit the impact that these protozoa may have on profitable production, are monitoring of water run-off, checking the condition of livestock water sources and providing both a high level of nutrition and protein to livestock. A high plane of protein in the diet has been reported to enhance the immune responses of lambs, preventing strongylid worms establishing infection (Sykes and Greer, 2003; Greer, 2008) and also reported for protozoa in intensively raised livestock (Olson et al., 1995; Olson et al., 2004). This will help limit the number of lambs acquiring protozoan infections and enhance the strength of their immune response to resist the onset of infection.

Although measurements post-mortem were unable to be conducted in these studies, the inflammatory and immune responses evoked by lambs in an attempt to counteract protozoan infections, were likely to have influenced the percentage weight of gastrointestinal tract organs (weight of organs relative to the overall live weight). Such increased weights of gastrointestinal organs have been previously reported for strongylid parasites (Liu et al., 2003; Jacobson et al., 2009c). In Chapter Ten, one lamb flock was exposed to a higher, natural strongylid larvae challenge and had lower dressing percentages when compared to a flock which was exposed to a lower larvae challenge, along with grazing pastures of poorer quality and quantity. The reported inflammatory and immune response attributed to protozoan parasites (Snodgrass et al., 1984; Scott et al., 2002; Buret et al., 2003), were likely to have reduced feed intake, digesta flow and feed...
conversion efficiency of infected lambs, although in these on-farm grazing studies, these measurements impractical to obtain in grazing lambs managed under commercial extensive conditions.

Adjusted, log-transformed mean WECs in both Chapters Nine and Ten were not significantly correlated with any carcase attributes. More in-depth research and analyses examining the potential impacts of strongylid, protozoan and bacterial species on carcase attributes, would be necessary to determine if pathogenic species have a negative impact on carcase attributes.

A small number of previous studies have attempted to examine whether protozoan parasites impact negatively on production attributes of lambs (Snodgrass et al., 1984; Olson et al., 1995). These studies adopted the practice of experimentally barn raised germ/pathogen free lambs with protozoa and some negative impacts on production were observed. These included reduced feed intake, reduced feed efficiency, slower growth rates and lighter live weight (Snodgrass et al., 1984; Olson et al., 1995). In Chapters Eight and Nine, detection of protozoa was associated with lighter live weights, but these findings were inconsistent across different sampling occasions. In Chapters Eight and Nine, detection of protozoa in young lambs (<2 months old) was associated with reduced live weights and growth rates. Clinical signs of protozoan infections have been reported to be routinely observed in young livestock, which may explain to why differences in live weight were observed in young lambs in Chapters Eight and Nine. However, as these studies were conducted on-farm and many uncontrollable variables may have influenced live weight measurements, some uncontrollable variables potentially contributed to inconsistent associations between protozoan detection and observed live weight. Some of these
uncontrollable variables include feed intake, gut fill, time off feed (Thompson et al., 1987; Warriss et al., 1989) and lamb birth type (single or twin) (Kenyon et al., 2004; Hatcher et al., 2009).

11.1.2 ASSOCIATIONS BETWEEN PHENOTYPIC FAECAL TRAITS AND MOLECULAR PARASITE DETECTION

With diarrhoea a widespread problem for sheep enterprises worldwide, a cross-sectional epidemiological survey was conducted by questionnaire and the results reported in Chapter Four. This survey identified a high (64.8%) prevalence of reporting recent or active outbreaks of diarrhoea within meat lamb flocks across southern WA. With such a high number of properties reporting diarrhoea (particularly in a significantly drier than normal season [2010]), an investigation was conducted into the association between faecal attributes and molecular parasite detection in Chapters Eight, Nine and Ten.

In both Chapters Eight and Nine, Cryptosporidium-positive lambs in all four flocks studied had an increased risk (2.8–11.6 times) of producing non-pelleted faeces, when compared to negative lambs. Giardia-positive lambs also had an increased risk (2.4–14.0) of producing non-pelleted faeces, this was observed in only three of the four flocks studied. Faecal dry matter percentages were lower and FCSs higher for lambs positive for Cryptosporidium or Giardia in both Chapters Eight and Nine. The increased risk of loose, non-pelleted faeces was most commonly detected in lambs 3 months old or younger. The formation of breech fleece faecal soiling and an increased risk of lambs predisposed to blowfly strike is strongly linked to diarrhoea (Broughan and Wall, 2007). Protozoa-positive lambs in Chapter Nine had an increased risk of moderate to severe breech fleece faecal tra...
soiling. Breech fleece faecal soiling scores were not recorded for two flocks in the study reported in Chapter Eight.

Strongylid WECs were positively correlated with FCSs and negatively correlated with FDM% for all flocks studied in Chapters Eight and Nine. In Chapter Eight, *Trichostrongylus* spp. – positive lambs were reported to also have an increased risk (6.6–8.3 times) of producing non-pelleted faeces in both flocks. Furthermore, a high number of internal parasite species detected per lamb (both strongylids and protozoa) was associated with more loose, non-pelleted faeces in both these flocks. In Chapter Ten, a greater pasture larval challenge (detected by both traditional and molecular diagnostics) was found to significantly increase FCSs (first and second samplings), decrease FDM% (second and final samplings) and increase breech fleece faecal soiling scores (second and third samplings).

Although diarrhea in lambs is routinely attributed to strongylid worms (Besier and Love, 2003; Sargison, 2004), protozoa have been reported to be another pathogenic factor associated with diarrhea (Snodgrass *et al.*, 1984; Xiao *et al.*, 1994; Olson *et al.*, 1995; Aloisio *et al.*, 2006). *Trichostrongylus* spp. is typically referred to as the ‘black scour worm’ and a common cause of diarrhea in lambs (Besier and Love, 2003; Woodgate and Besier, 2010). The results from Chapter Eight concur with previous findings related to this strongylid species. However, diarrhea in lambs has been ascribed to many different non-pathogenic and pathogenic factors, thus the condition is considered to be multi-factorial (Sargison, 2004). The findings that detection of protozoa was associated with loose, non-pelleted faeces, is consistent with previously published literature (Olson *et al.*, 2004; Aloisio *et al.*, 2006; Díaz *et al.*, 2010). However, there is little previous research examining the
concurrent roles of both strongylid and protozoan parasites as potential risk factors for diarrhoea. Therefore, future research into diarrhoea and breech fleece faecal soiling in lambs needs to consider both strongylid and protozoan parasites as potential contributing causes of diarrhoea in extensive grazing lambs (particularly those lambs 2 months old or younger).

11.1.3 INTERNAL PARASITE EPIDEMIOLOGY AND PUBLIC HEALTH RISKS

The reported overall prevalences of Cryptosporidium and Giardia were high in Chapters Seven, Eight and Ten. Over 80% of lambs in one flock tested positive for Cryptosporidium on at least one occasion for one flock studied in Chapter Seven. The individual sampling point prevalences in Chapters Seven, Eight and Ten were within similar ranges to other recent epidemiological studies conducted in the USA (Santin et al., 2007), Europe (Gómez-Muñoz et al., 2009; Robertson et al., 2010) and Australia (Yang et al., 2009; Nolan et al., 2010). In these three chapters, Cryptosporidium point prevalences were routinely higher than Giardia point prevalences, which has previously been reported in Australia (Ryan et al., 2005) and the USA (Santin et al., 2007). The prevalences of Cryptosporidium and Giardia in lambs sampled at lairage (Chapter Seven 29.4–42.0% and Chapter Ten 13.9–21.6%) were not significantly different to the on-farm sampling prevalences (Chapter Seven 18.5–42.6% and Chapter Ten 7.5–25.5%). The highest protozoa prevalences were detected when lambs were ~2 months old (second sampling) and ~7–8 months old (final sampling) in Chapters Seven and Ten.

The most common Cryptosporidium species detected in lambs in Chapters Seven, Eight and Ten were C. ubiquitum and C. xiaoit, with C. parvum the most frequently detected
species from one flock in Chapter Eight. *Cryptosporidium ubiquitum* was the most commonly detected species in young lambs, however *C. xiao* was the most frequently detected species, particularly in older lambs when sampled. In Chapters Seven and Ten, *Cryptosporidium parvum* was more routinely detected in lambs (more widespread in older lambs) and was also a common species found in mixed infections (*C. xiao* and *C. parvum*). *Giardia duodenalis* assemblage E was the most frequently detected genotype in lambs from Chapters Seven, Eight and Ten, although assemblage A was more frequently detected than assemblage E in one flock in Chapter Eight. Assemblage A was more routinely identified from lambs as they aged and in mixed assemblage infections (assemblages A and E). In general, the protozoan species/genotypes isolated from lambs in the studies reported in Chapters Seven and Ten, were of low zoonotic potential. High proportions of *C. parvum* were identified from one flock in Chapter Eight and high frequencies of assemblage A were identified from one flock in Chapter Eight and from both flocks in Chapter Ten.

The survey reported in Chapter Four found that the water source was a potential risk factor, contributing towards outbreaks of diarrhoea within meat lamb flocks. As a result, water samples were collected and tested from sheep water sources in Chapters Seven and Ten. *Cryptosporidium* was detected in water samples from one flock in Chapters Seven and one flock in Chapter Ten. *Giardia* was only identified from one flock (Chapter Ten). By using sequence analysis of protozoan parasite DNA amplified from faecal genomic DNA extracts, the genetic similarity of isolates detected from water and faecal samples were compared. In both Chapters Seven and Ten, these analyses showed that many of the *Cryptosporidium/Giardia* isolates detected from lambs were identical to those *Cryptosporidium/Giardia* isolates identified from water sources. It is possible following
heavy rainfall, that sheep faeces containing (oo)cysts were washed into the open dams and potentially contributed to some lambs acquiring a protozoan infection. Protozoa were only isolated from open dam water sources in Chapters Seven and Ten. The water tested from both soaker catchments in a running river/creek and from troughs sourcing water from a dam by underground pipes, never tested positive for any protozoa. An opportunity exists to further enhance our knowledge of protozoa transmission cycles in livestock and the risk of water contamination with pathogenic and non-pathogenic infectious agents.

Nearly all overall strongylid prevalences (lambs positive for at least one strongylid species across the five samplings) were close to 100% in Chapters Seven and Ten, with infection detected by both conventional techniques and qPCR. In Chapters Five, Six and Ten, significant falls in strongylid prevalences were observed in sampling periods after an anthelmintic treatment had been administered to lambs. With all flocks grazing pastures that were contaminated with strongylid larvae, it was expected that overall strongylid prevalences would be high. In the six flocks described in Chapters Five, Six, Eight and Ten, *T. circumcincta* and *Trichostrongylus* spp. were the most commonly detected strongylid species, with *C. ovina* and *Oesophagostomum* spp. identified less frequently. *Haemonchus contortus* was only detected in 3 out of the 6 flocks studied, which were located in Frankland and Boyup Brook. The climate conditions experienced by these farms are favourable for the survival and transmission of *H. contortus* (Besier and Dunsmore, 1993a, b), because they are more likely to receive summer rainfall. With ~75% of farmers reported to have administered an anthelmintic to their meat lamb flocks (Chapter Four), the development and availability of molecular diagnostics to differentiate strongylid species, would improve the selection of specific anthelmintic treatments (targeted treatment of highly pathogenic *H. contortus*). Furthermore, molecular techniques may play a role in...
monitoring anthelmintic treatment efficacies. This would assist sheep enterprises in their selection and cycling of different anthelmintic treatment compounds, to minimise the development of anthelmintic resistance.

11.1.4 COMPARING MOLECULAR AND TRADITIONAL METHODS FOR THE DETECTION OF INTERNAL PARASITES

In Chapters Five and Six the levels of agreement between traditional WECs and conventional PCRs for detecting patent strongylid infections are reported. Strong overall levels of agreement (>90%) were observed between traditional and molecular results, with strongylid eggs considered to be the likely main source of strongylid DNA present in the genomic DNA extracted directly from faeces. This strong level of agreement across a variety of successive samplings (lambs of different age and acquired immunity status), together with the positive correlations observed between WEC and the number of strongylid species detected per lamb by PCR, support the hypothesis that worm eggs are the major strongylid DNA source. In Chapter Ten, species specific qPCRs were used to screen genomic DNA extracted directly from faeces. Spiked analyses showed that qPCRs had greater detection sensitivities when compared to conventional PCR. In Chapter Ten, the overall level of agreement between qPCR and WEC was moderate (77%) and more variable than observed in Chapters Five and Six. This suggests that although the qPCRs offered greater sensitivity, they potentially detected non-patent sources of strongylid DNA and contributed to false positive identification of some strongylids.

A new molecular method was developed and trialled, whereby a modified strongylid larvae recovery procedure recovered larvae which had been floated off pasture following soaking. Genomic DNA was extracted from the recovered material and then screened with
strongylid species-specific qPCRs and conventional PCRs. Although the levels of agreement between molecular and pasture larval count results were moderate (67%), the molecular technique has the potential to be further developed to improve the recovery rate of larvae. Utilising molecular techniques offer significant advances for routine veterinary and human diagnostics, particularly through limiting the intensive labour input and processing time to achieve results. The most important area for further research into assessing whether molecular tests can be implemented and incorporated into routine diagnostic, is an investigation into their cost-benefits and practical implications for diagnostic laboratories, combined importantly with a forecasted uptake and probable demand by livestock enterprises.

11.2 FUTURE WORK

There are a number of subject areas identified as a result of this research that require further investigation. Further examination into internal parasite species/genotype epidemiology and their demographics across different regions throughout different sheep rearing regions of Australia is required. Even though this research provided insight into strongylid nematode and protozoan prevalences using molecular diagnostic techniques, it was only conducted on five farms, which were all located in agriculture zones two or three in WA. There is the potential for a larger scale research study, to determine whether (1) different geographical areas have different species/genotypes with respect to internal pathogens (parasites, bacteria and viruses) and also (2) if internal pathogen detection is associated with reduced lamb productivity. Such bacteria as *Salmonella*, *Campylobacter jejuni*, *Yersinia* and *Chlamydophila* have all been reported to impact lamb productivity, however little current research exists on these bacteria in sheep flocks.
The final experiment in Chapter Ten highlighted the potential for the development of a large scale study focussed on exploring a broad variety of internal pathogens (parasites, bacteria and viruses) in a number of different sheep enterprises across Australia. The development and testing of multiplex qPCR assays capable of detecting such pathogens would increase sample throughput and enhance the knowledge on the prevalence of these potential pathogens. There are still significant gaps in the knowledge regarding the prevalence of bacteria and virus in sheep and lamb enterprises, both on-farm and in lairage within Australia. Such examples of these gaps in knowledge include the prevalence of these pathogens across different geographical locations across Australia, differences between on-farm and lairage prevalences and potential risk of contamination of human food or water sources. Furthermore, there is little current research documenting the consequences such pathogens may have on lamb productivity performances or the risk of diarrhoea. There is potential for a research study to provide multiple pathogen analyses in order to determine or examine, how or why certain pathogens are associated with reduced lamb productivity performances.

The red legged earth mite (*Halotydeus destructor*) is a major pest of pasture legumes in the winter rainfall areas of southern Australia and although it has not been included in any research associated with lamb or sheep productivity, it potentially has negative impacts on lamb faecal consistency, as well as pasture growth (Ridsdill-Smith *et al.*, 2005). The red legged earth mite is the most abundant pasture pest, particularly during the winter months when their numbers are highest and often the mites or eggs are consumed by grazing sheep (Ridsdill-Smith *et al.*, 2005). There have been no reports of the mites or their eggs linked with an increased risk of diarrhoea or reduced flock productivity performance in lambs or sheep, although mite eggs are passed in sheep
faeces and often are observed during light microscope analyses. Therefore investigation is warranted to examine their distribution and prevalence across different geographical regions in southern WA, with the potential to develop a PCR capable of detecting these mites and to aid in determining whether their detection is potentially associated with increased FCS, decreased FDM% and reduced lamb productivity performances.

Feeding intake could not be recorded in the studies described, however future research could examine whether single or mixed internal parasite infections (strongylid nematodes and protozoa) impact differently on feed consumption by lambs. This would require managing sheep intensively and this may allow for such a study to also ascertain whether specific new anti/protozoal chemotherapeutic agents (e.g. protein disulfide isomerases, nitazoxanide, halfuginone lactate and cyclodextrin) are cost-effective against Cryptosporidium and Giardia infections. Such a trial would allow feed intake to be controlled and therefore limit the variability in animal live weight which is associated with the gastrointestinal contents and the time animals are held off feed. It would be important that such a trial examines the possible impacts that single or mixed internal parasite infections have on on-farm and off-farm profitable productivity; including feed consumption, carcase attributes (HCW, dressing percentage and GR knife fat depth), production performance attributes (live weight, growth rate and body condition score) and faecal attributes (FCS, FDM% and breech fleece soiling score).

Although a high digesta water content and rapid flow rate through the small and large intestines has been reported to reduce faecal pellet formation within the spiral colon of the intestine (Reid and Cottle, 1999; Waghorn et al., 1999), our understanding of faecal formation physiology is limited. The survey questionnaire of lamb meat producers in
Chapter Four identified flock water source as a major risk factor for both diarrhoea and breech fleece faecal soiling. There is a need to examine the pathogenic (parasites, bacteria and viruses) and non-pathogenic (fertilisers, chemicals and heavy metals) factors associated with water contamination and diarrhoea, along with exploration into water run-off from pastures into livestock water sources. Different water sources (dam, river, bore and scheme) could be tested across a variety of different geographical locations to determine if they are positive for protozoa and whether the species/genotypes are of livestock or human origin.

Even though the molecular diagnostic techniques utilised in this research to detect strongylid nematode infections had a high level of agreement with traditional techniques (light microscopy), it is critical that these techniques are tested against larval cultures (on individual faecal samples), total worm counts and histopathology tissue sections. This would definitely confirm whether established internal parasite infections (strongylid worms or protozoa) were detected, rather than eggs, (oo)cysts or larval tissues simply passing through the animal and being expelled in their faeces. Furthermore, the molecular techniques utilised in this study were strongly dependent on the genomic DNA obtained from faecal DNA extractions. The PCR and qPCR results would be significantly influenced by the biomass of each parasite within a faecal sample, along with the time between sample collection and DNA extraction. It is necessary to compare the DNA obtained from samples from the different life cycle phases of internal parasites (particularly the egg, $L_1$, $L_2$, $L_3$, $L_4$, and the adult form of strongylid worms) and also DNA yield from samples of different ages (time in days between sampling and laboratory processing i.e. does time differences between sample processing influence DNA yield).
Finally, the molecular pasture larvae method developed in Chapter Ten potentially offers diagnostic laboratories a rapid method capable of detecting and quantifying the species and number of strongylid larvae present on pasture samples. This is potentially highly advantageous for those producers striving to limit the strongylid larvae contamination of pastures before lambing and to reduce the risk of highly pathogenic larvae (H. contortus) accumulating on pastures and infecting growing lambs. Furthermore, a fast, reliable diagnostic technique with minimal labour input and a reduced processing time, would be attractive for both diagnostic laboratories and agricultural agencies. More work is required to further test and validate this molecular technique, while there is further opportunity for testing new molecular techniques to compare with traditional diagnostic techniques that are time consuming, labour intensive and potentially generating unreliable, biased results.

11.3 CONCLUSIONS

The thesis has addressed the impact that internal parasites (strongylid worms and protozoa) have on lamb productivity performance. This research was designed to utilise innovative, molecular diagnostic tools to identify sheep positive for different species of internal parasites. These results were then used to assess whether molecular detection of internal parasites, were associated with reduced production performances in lambs, extensively managed on commercial sheep enterprises. This was performed by conducting statistical analyses on growth and carcase attributes of lambs to assess whether infection was associated with effects on production attributes for lambs. Significant differences were observed consistently for carcase and faecal attributes, with infected lambs having lower HCWs, lower dressing percentages and more loose, wetter faeces, than uninfected lambs. This research also provided epidemiological information on the changing protozoan
parasite prevalences and demographics from lambs of different ages. It also highlighted that those protozoan parasites can be found in livestock water sources and require monitoring in order to limit the possibility of contamination of human drinking water sources. This research provided producers with new information towards internal parasitism within extensively grazing lamb flocks, establishing that protozoa detection was associated with reduced carcase productivity performances.
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CHAPTER 13: APPENDICES

13.1 APPENDIX ONE: QUESTIONNAIRE SURVEY

The cover letter and survey questionnaire described in Chapter Four are shown on the following pages.
Internal parasites and scouring survey: WA 2010

Dear Sheep Producer

My name is Josh Sweeny and I am a post-graduate student at Murdoch University working on a project investigating the role internal parasites play in diarrhoea (scouring) in lambs. The project is being carried out under the supervision of Associate Professor Una Ryan, Professor Kevin Bell, Dr Caroline Jacobson and Professor Ian Robertson (Dean of Murdoch Veterinary School). This survey is one part of the project to determine background information on lamb scouring and internal parasites.

The aims of this survey are to determine:
  ➢ if scouring is an important issue for prime lamb farmers in WA?
  ➢ what are the important risk factors associated with scouring in young sheep?
  ➢ what control measures farmers are currently using?

The survey consists of 20 questions over 2 pages and should take approximately 5 minutes (of your time) to complete.

This study has been approved by the Murdoch University Human Research Ethics Committee (Approval 2009/222). If you have any reservation or complaint about the ethical conduct of this research and wish to talk with an independent person, you may contact Murdoch University’s Research Ethics Office (Tel. 08 9360 6677 or e-mail ethics@murdoch.edu.au). Any issues you raise will be treated in confidence and investigated fully and you will be informed of the outcome. There is some space at the end for you to make any comments that you feel may be important or relevant.

If you have any questions, please call Joshua Sweeny at Murdoch University on 9360 2495 or e-mail J.Sweeny@murdoch.edu.au.

All information is strictly confidential.
Please send replies in the self addressed envelope supplied to:
  Joshua Sweeny
  Division of Veterinary and Biomedical Sciences
  Murdoch University
  South Street
  Murdoch WA 6150
Or fax replies to (08) 9360 6628

Participant consent
I have read the Information letter about the nature and scope of this survey. Any questions I have about the research process have been answered to my satisfaction. I agree to take part in this research. By submitting this survey I give my consent for the results to be used in the research. I am aware that this survey is anonymous and no personal details are being collected or used. I know that I may change my mind, withdraw my consent, and stop participating at any time; and I acknowledge that once my survey has been submitted it may not be possible to withdraw my data.

I understand that all information provided is treated as confidential by the researchers and will not be released to a third party unless required to do so by law. I understand that the findings of this study may be published and that no information which can specifically identify me will be published.

Signed:               Date:               Name:
PARASITES AND SCOURING SURVEY: WA 2010

1. Location (shire):

2. Average annual rainfall:
   _______mm or _______ inches
   _______ hectares or _______ acres

3. Area in crop 2010:
   _______ hectares or _______ acres

4. Area grazed 2010:

5. Number of ewes joined in 2010:

6. Number of ewes joined to terminal sires in 2010:

7. What month did lambing commence for your prime lambs in 2010?

8. Do you run cattle on your property?
   [ ] Yes   [ ] No

9. Did the 2010 prime lamb drop receive a drench at, or after weaning?
   [ ] Yes   [ ] No

10. Were the ewes (dams of prime lambs) drenched in the 2 months before lambing?
    [ ] Yes   [ ] No

11. Do you use faecal worm egg counts to decide if sheep require drenching?
    Never    Occasionally    Usually    Always
    [ ]       [ ]            [ ]

Scouring (Diarrhoea) and dag in lambs

12. Have you seen any scouring in your prime lambs in 2010?
    [ ] Yes   [ ] No

13. If you observed scouring in your prime lambs, in what month did this first occur?
14. What % of your 2010-drop prime lambs would you estimate to have experienced scouring or been affected with moderate-severe dags (scores 3, 4 or 5 on the scale below)?

15. If approximately 5% of your prime lambs were scouring, would you…

<table>
<thead>
<tr>
<th>Monitor to see if situation becomes worse</th>
<th>Conduct worm egg count on mob</th>
<th>Drench whole mob</th>
<th>Drench scouring lambs</th>
<th>Have problem investigated by a vet</th>
<th>Do Nothing</th>
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16. If approximately 25% of your prime lambs were scouring, would you…

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<tr>
<th>Monitor to see if situation becomes worse</th>
<th>Conduct worm egg count on mob</th>
<th>Drench whole mob</th>
<th>Drench scouring lambs</th>
<th>Have problem investigated by a vet</th>
<th>Do Nothing</th>
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17. If approximately 50% of your prime lambs were scouring, would you…

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<tr>
<th>Monitor to see if situation becomes worse</th>
<th>Conduct worm egg count on mob</th>
<th>Drench whole mob</th>
<th>Drench scouring lambs</th>
<th>Have problem investigated by a vet</th>
<th>Do Nothing</th>
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18. What were the sources of water available to the 2010 drop of prime lambs (please tick all water sources, if more than one was available to the lambs)?

- Dam
- River/creek
- Troughs filled from bore
- Troughs filled from dam/river
- Trough filled with scheme water

19. Are you aware of coccidian parasites causing disease in sheep in your area?

- Yes
- No
- Unsure
20. Please indicate if you are aware of the following coccidian parasites infecting/or causing disease in sheep on broad-acre grazing enterprises in Western Australia (please tick all parasites if you have heard more than one):

- *Eimeria*
- *Cryptosporidium*
- *Giardia*

Thank-you for taking the time to complete this questionnaire.

If you have any further comments, please feel free to write them in the space below of this page.

Should you want the analysis, results and feedback from this survey and project, please include your name and contact details, along with any other comments or questions below.

Contact Details (if you choose), comments and questions list below.
13.2 APPENDIX TWO: MODIFICATIONS TO FAECAL DNA EXTRACTION

Genomic DNA was extracted directly from 200 – 300mg crude faecal samples using a Power Soil DNA Kit (MolBio, Carlsbad, CA), with modifications to the manufactures protocol are included in bold text below:

1. Label 4 x 24 2ml tubes
2. Label 1 x 24 Filter tubes
3. Add 60 μL C1 to 24 labelled PowerBead tubes

4. **Heat the PowerBead tubes filled with C1 solution**
5. Add a small sample (0.25-0.3g) of faeces to each of the PowerBead tubes
6. **Raise heating temperature to 100 °C and heating for 5 mins**
7. **Increase vortex time to 5 mins**
8. **The freeze-thaw cycle process of freezing the PowerBead tubes of faecal samples in liquid nitrogen for 5 mins and then heating the samples at 95°C for 5 mins was performed (Geurden et al. 2008; Thompson et al. 2009). There was a total of five freeze thaw cycles performed to aid destruction of the resistant oocysts.**
9. **Heat for 8 mins at 100 °C**
10. Vortex for 5 mins (Max Mix Speed)
11. Add 250 μL of C2 to clean 2ml Collection tubes
12. Centrifuge PowerBead tubes at 10,000 x g for 30 s
13. Pour the supernatant from PowerBead tubes to the C2 filled Collection tubes.
14. Vortex each tube for 10secs, Incubate at 4 °C in fridge for 5 mins
15. Centrifuge the tubes at 10,000 x g for 1 min
16. Pour into clean 2ml Containing 200µL of C3
17. Vortex each tube for 10 secs. Incubate at 4 °C in fridge for 5 mins
18. Centrifuge the tubes at 10,000 x g for 1 min.
19. Add 1000µL of C4 to clean tubes. (Transfer no more than 650µL)
20. Using a pipette transfer supernatant into clean 2ml C4 filled collection tubes
21. Load 650µL onto a Spin filter tube, centrifuge for 1 min and discard the flow through.
   Repeat the above process to achieve a total of three loads for each sample (3 centrifuge runs)
22. Add 500µL of C5 to empty Spin Filter tubes and centrifuge for 60 s
23. Discard flow through
24. Centrifuge again for 90 s
25. Carefully remove Spin Filters from tubes and place in clean Collection tubes (avoid splashing any remaining C5 solution on filter)

26. Add 50µL instead of 100µL of C6 to the centre of the white filter membrane.
27. Centrifuge for 60 s
28. Discard the Spin Filter and the DNA is ready for downstream application or to be set in a −20°C Freezer.
Figure 13.1: Graphical representation of the Power Soil DNA Isolation Kit methodology.
Figure 13.2: Alignment of 5.8S, ITS-2 and partial 28S nuclear ribosomal DNA sequences, and location (grey) of individual forward primers (HAE, TEL, TRI, CHO, OEV), designed to the ITS-2 and evaluated in silico for specificity. Primer NC2 (reverse) is located in the 5' region of the 28S rRNA gene. Polymorphic positions indicated with International Union of Pure and Applied Chemistry (IUPAC) codes (Bott et al., 2009).
13.4 APPENDIX FOUR: SPIKE ANALYSES AND PCR ASSAY MINIMUM DETECTION LIMITS

Figure 13.3: *Cryptosporidium* spike analysis, PCR products from multiple *Cryptosporidium* species isolated at the 18S rRNA gene (~540bp). *C. hominis, C. parvum, C. melagridis, C. ubiquitum, C. felis, C. xiaoi, C. canis, C. suis, C. baileyi, C. wriairi, C. ryanae, C. andersoni* and *C. bovis* spiked samples (lanes 1 – 14) and negative control (lane 15) on a 1.0% agarose gel stained with SYBR® Safe DNA stain (Invitrogen, USA).

Figure 13.4: *Cryptosporidium* spike analysis, PCR products from multiple *Cryptosporidium* species isolated at the actin gene (~830bp). *C. hominis, C. parvum, C. ubiquitum, C. xiaoi, C. suis, C. baileyi, C. andersoni* and *C. bovis* spiked samples (lanes 1 – 8) and negative
control (lane 9) on a 1.0% agarose gel stained with SYBR® Safe DNA stain (Invitrogen, USA).

**Figure 13.5:** *Giardia duodenalis* spike analysis, PCR products from multiple *Giardia* assemblages isolated at the *gdh* gene (~830bp). Assemblages A, B, C, D, E, F and G spiked samples (lanes 1 – 7) and negative control (lane 8) on a 1.0% agarose gel stained with SYBR® Safe DNA stain (Invitrogen, USA).

**Figure 13.6:** *Giardia duodenalis* spike analysis, PCR products from multiple *Giardia* assemblages isolated at the β-*Giardin* gene (~511bp). Assemblages A, B, C, D, E and F spiked samples (lanes 1 – 6) and negative control (lane 7) on a 1.0% agarose gel stained with SYBR® Safe DNA stain (Invitrogen, USA).
Figure 13.7: Example of serial dilutions to determine the minimum amount of DNA for successful PCR amplification for *Trichostrongylus* spp. minimum DNA detection limit by PCR, PCR products at the IT-S nuclear rDNA gene (~268bp). Varying genomic DNA/µL concentrations 10,000, 5000, 1000, 500, 100, 50, 25, 15, 10, 5, 2, 1 and 0.1 pg/µL (lanes 1 – 13) on a 1.0% agarose gel stained with SYBR® Safe DNA stain (Invitrogen, USA).

Figure 13.8: *Salmonella* spike analysis, PCR products isolated at the *ompF* gene (~578bp). Spike samples (lanes 1 – 10), negative control (lane 11) and positive control (lane 12) on a 1.0% agarose gel stained with SYBR® Safe DNA stain (Invitrogen, USA).
Figure 13.9: *Campylobacter jejuni* spike analysis, PCR products isolated at the 16S rRNA gene (~287bp). Spike samples (lanes 1 – 14) and negative control (lane 15) on a 1.0% agarose gel stained with SYBR® Safe DNA stain (Invitrogen, USA).
13.5 APPENDIX FIVE: qPCR RESULTS

13.5.1 CRYPTOSPORIDIUM PARVUM DIAGNOSTIC LOCUS

Figure 13.10: qPCR analysis for confirmation of mixed Cryptosporidium species infections at the diagnostic locus (unique Cryptosporidium-specific protein coding gene) showing samples identified as C. parvum positive (Morgan-Ryan et al., 1997; Yang et al., 2009).
13.5.2 qPCR FOR STRONGYLID SPECIES DETECTION

Figure 13.11: qPCR analysis for detection of *Teladorsagia circumcincta* at the ITS-2 gene.

The DNA was extracted directly from lamb faeces and material recovered from the modified pasture larvae recovery procedure.
13.6 APPENDIX SIX: SHEEP GENOTYPE I SEQUENCES

13.6.1 18S rRNA:

GenBank Accession Number: HQ317903

5' CCT ACC GTG GCA ATG ACG GGT AAC GGG GAA TTA GGG TTC GAT TCC GGA GAG GGA GCC TGA GAA ACG GCT ACC ACA TCT AAG GAA GGC AGC AGG CGC GCA AAT TAC CCA ATC CTA ATA CAG GGA GGT AGT GAC AAG AAA TAA CAA TAC AGG ACT TTA AAT AGT TTT GTA ATT GGA ATG AGT GAA GTA TAA ACC CCT TTA CAA GTA TCA ATT GGA GGG CAA GTC TGG TGC CAG CAG CCG CGG TAA TTC CAG CTC CAA TAG CGT ATA TAA AAG TTG TTG CAG TTA AAA AGC TCG TAG TTG GAT TTC TGT TTA TTA TTT ATA ATG AAT ATT TTA TTA ATA TTA TTA ATA TAA TAT TAA CAT AAT TTA AAT TTT AGT ATT TTA TAG TAT ATG A 3'

13.6.2 ACTIN:

GenBank Accession Number: HQ317904

5' CAC GAC CGT GGC TAC AGT TTC ACA ACT ACA GCT GAG AGA GAA ATA GTA AGA GAT ATT AAA GAA AAG CTT TGT TAC ATT GCT CTT GAT TAT GAG GAA GAA ATG AAA AAA TCT CAG GAG TCT TCT GAA TTA GAA AAG ACA TAT GAA CTA CCT GAC GGT CAT GTA ATT ACC GTA GGC AGT GAA AGA TTA CCA CCA GAG GCT CTA TTC CAA CCA GGT CTT TTA GGT AAA GAA GCT GTT GGT ATC GGT GAG CCT GCT ATT CCA ATC TAT CAT GAA ATG TGG AGA AGA TGG C 3'
13.6.3 ACTIN PROTEIN SEQUENCE:

5' HDRGYSFTTTAEREIVRDIEKLICYIALDYEEEKKKSKQESSE
LEKTYELPDBGHVTVGSERFRCPAEALFQPGLLGKEAVGIGEPA
IPIYHEMWRRW 3'