Immune Responses in Dairy Cattle Naturally Exposed to *Fasciola hepatica*

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Abstract

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Fasciola hepatica is a parasitic trematode capable of infecting a range of vertebrate species including livestock and humans. Both clinical disease (fasciolosis) and sub-clinical infections are of major economic and welfare importance in food producing animals. The life cycle of *F. hepatica* requires an intermediate host, which in the UK is the mud snail *Galba truncatula*. High levels of moisture and ambient temperatures between 10-30^{o}C provide optimal conditions for the development of both parasite and snail. As a consequence of changing weather patterns, disease prevalence has increased in recent years, whilst an over-reliance on effective anthelmintics to control disease has resulted in the selection of drug resistance within parasite populations. Studies investigating vaccination as a potential method to control *F. hepatica* in cattle have identified specific components of the vaccine-induced immune response such as IgG2 antibody titre and avidity, which are associated with protection. Conversely, evidence from experimental infections indicate that *F. hepatica* modulates the host immune system towards a non-protective type-2 response, extending parasite survival within the host.

The overall objective of this thesis was to analyse the immune response in calves and adult cattle naturally exposed to, and infected with, *F. hepatica*. This was achieved firstly through the validation of a herd level diagnostic test to identify infected beef and dairy herds, and secondly by evaluating the type of immune response present in infected animals. The outputs will be valuable in informing vaccine development, since the type of immune response present in naturally infected cattle, will ultimately have implications for how such vaccines are applied in the field.

Chapter 3 describes the validation of a composite sample analysis for fluke egg counts. A total of 138 individual samples from 7 commercial beef herds in mid-Wales were sampled. Fluke egg counts were done on individual samples in addition to composite samples composed of ten 5g samples. These data together with individual counts from a further 22 dairy farms (638 individual samples) were fitted to negative binomial distributions at the farm level. These were stochastically re-sampled to generate a range of predicted composite counts from which confidence intervals and test sensitivity were determined. When referred back to the original counts, all composite counts were within the generated confidence intervals, with the lower confidence interval indicating a 95% test sensitivity at ≥0.4 eggs per gram of faeces compared to individual count data. With the exception of lactating dairy cattle, diagnosis of *F. hepatica* is

limited to individual faecal and/or serum sampling. This analysis represents an important development, since a validated composite worm egg count for cattle provides a simple yet effective test for screening groups of animals for infection.

In chapter 4, immune responses in naïve dairy heifers (n=42) naturally exposed to *F. hepatica* were evaluated. Calves on 3 commercial UK dairy farms were sampled monthly over the course of their first grazing season and analysed to determine fluke infection status and parasite specific immune responses. Where infection was present, this was associated with increases in type-2 associated responses, with increases in interleukin-4 production, interleukin-5 transcription and an eosinophilia. A reduction in the type-1 associated cytokine, interferon-γ was also observed over the course of infection. These findings suggest that a natural challenge with *F. hepatica* induces a non-proliferative type-2 response. This has implications for vaccine development and application, since current evidence suggests that stimulation of additional components such as IgG2 antibody and strong cell mediated responses are required for protection.

Chapter 5 describes a study carried out on a commercial dairy farm, characterising the immune responses in adult dairy cattle (n=27) with chronic infections. The effect of treating infected animals with triclabendazole (12mg/kg) on the immune response was also assessed. Both parasite specific and mitogen stimulated interleukin-4 production were positively associated with *F. hepatica* antibody titres based on linear regression analysis, whilst no such correlation was found with interferon-γ. This suggests that modulation of the immune response towards a type-2 response is a feature in chronic infections. Additionally, increases in the regulatory cytokines Transforming Growth Factor-β and interleukin-10, associated with infection in pre and post treatment groups respectively may indicate that these cytokines play a role in parasite induced immune modulation, which has been described previously in experimentally infected cattle.

Overall, these results show that cattle exposed to and infected with *F. hepatica* under natural grazing conditions develop a type-2 immune response. This has implications for future vaccination programmes, as the presence of immune modulation arising from natural infection suggests any vaccine induced immune response should be fully developed prior to natural exposure to ensure protection. These results also highlight the importance of the impact of fluke infections on the host's immune system and the need to investigate and better understand the relationship between *F. hepatica* and other co-infecting pathogens.

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Dedication

I would like to dedicate this thesis to

Emma, Laura, John and Hannah,

Edie, Ina, Oliver and Iris.

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List of Abbreviations

(m)Ab	-	(monoclonal) Antibody
ΔAb	-	Change in Antibody
ADCC	-	Antibody Dependent Cell-mediated Cytotoxicity
AHVLA	-	Animal Health and Veterinary Laboratories Agency
AIC	-	Akaike Information Criterion
AMCase	-	Acidic Mammalian Chitinase
APCs	-	Antigen Presenting Cells
Arg1	-	Arginase-I
Az	-	Sodium Azide
BMT	-	Bulk Milk Tank
BSA	-	Bovine Serum Albumin
BVD	-	Bovine Viral Diarrhoea
Bϕ	-	Basophil
CD(4,8,…,*n*)	-	Cluster of Differentiation (4,8,…,*n*)
CDI	-	Centre for Dairy Information
CFSE	-	Carboxyfluorescein Succinimidyl Ester
CoAg	-	*F. hepatica* Copro-Antigen
ConA	-	Concanavalin A
COWS	-	Control Of Worms Sustainably
DALYs	-	Daily Adjusted Life Years
DCAR	-	Dropouts Completely At Random
DCs	-	Dendritic Cells
(c/g)DNA	-	(complimentary/genomic) Deoxyribonucleic Acid
DNase	-	Deoxyribonuclease
EBLEX	-	English Beef and Lamb Executive
EDTA	-	Ethylenediaminetetraaceitic Acid
ELISA	-	Enzyme-Linked Immunosorbent Assay
EU	-	Endotoxic Unit
E/S	-	*F. hepatica* Excretory/Secretory (products or antigen)
Eϕ	-	Eosinophil
FABP	-	*F. hepatica* Fatty Acid Binding Protein
FACS	-	Fluorescence-Activated Cell Sorting
FAO	-	Food and Agriculture Organisation of the United Nations
FCA	-	Freund's Complete Adjuvant
FCS	-	Foetal Calf Serum
FITC	-	Fluorescein Isothiocyanate
FIA	-	Freund's Incomplete Adjuvant
Fizz1	-	Resistin-like molecule alpha1
FoxP3	-	Forkhead box P3 molecule
FSC	-	Forward Scatter
GGT	-	Gamma-Glutamyltranspeptidase
GI	-	Gastro-Intestinal
GLDH	-	Glutamate Dehydrogenase
GST	-	Glutathione S-Transferase
HOL	-	Home Office License
HRP	-	Horse Radish Peroxidase
IFN-γ	-	Interferon-gamma
Ig(G,A,…,*x*)	-	Immunoglobulin (G,A,…,*x*)
IL-(1,2,4,…,*n*)	-	Interleukin-(1,2,4,…,*n*)
iNOS	-	inducible Nitric Oxide Synthase
LAMP	-	Loop Mediated Isothermal Amplification

LFH	-	Liver Fluke Homogenate
LPS	-	Lipopolysaccharide
LVPD	-	Liverpool Veterinary Parasitology Diagnostics
Lϕ	-	Lymphocyte
MAPKs	-	Mitogen-Activated Protein Kinases
MBP	-	Major Basic Protein
MCs	-	Mast Cells
MHC-I/II	-	Major Histocompatability Complex class-I/II
ML	-	Maximum Likelihoods
Mϕ	-	Macrophage/Monocyte
M1	-	Classically Activated Macrophage
M2	-	Alternatively Activated Macrophage
NADIS	-	National Animal Disease Information Service
NBD(s)	-	Negative Binomial Distribution(s)
NEJ(s)	-	Newly Excysted Juvenile fluke(s) (*F. hepatica*)
NO	-	Nitric Oxide
NP-40	-	Nonidet P-40 detergent
Nϕ	-	Neutrophil
OD	-	Optical Density
PAMPs	-	Pathogen-Associated Molecular Patterns
PBMC(s)	-	Peripheral Blood Mononuclear Cell(s)
(D-)PBS	-	(Dubelco's-)Phosphate Buffered Saline
(q)PCR	-	(quantitative) Polymerase Chain Reaction
pCO_2/O_2	-	Partial Pressure of Carbon Dioxide/Oxygen
PD-L2	-	Programmed Death Ligand 2
Pgp	-	P-glycoprotein
PHA	-	Phytohaemagglutinin
PP	-	Percent Positivity
PPD	-	Tuberculin Purified Protein Derivative
PRRs	-	Pattern Recognition Receptors
Prx	-	*F. hepatica* Peroxiredoxin
REML	-	Restricted Maximum Likelihoods
(m/t)RNA	-	(messenger/transfer) Ribonucleic Acid
RPMI	-	Roswell Park Memorial Institute
SI	-	Stimulation Index
SICCT	-	Single Intradermal Comparative Cervical Tuberculin (test)
SOCS(3)	-	Suppressor Of Cytokine Signalling (3)
SomAg	-	*F. hepatica* Somatic Antigen
SSC	-	Side Scatter
TCBZ	-	Triclabendazole
TCR	-	T-Cell Receptor
TegAg	-	*F. hepatica* Tegumental Antigen
TGF-β	-	Transforming Growth Factor-beta
$T_H1/2$	-	T-Helper 1/2
TLR(3,4,…,*n*)	-	Toll-Like Receptor (1,4,…,*n*)
TMB	-	Tetramethylbenzidine
TNF-α	-	Tumour Necrosis Factor-alpha
Treg	-	Regulatory T-cell
T0/1/2	-	Type-0/1/2 *F. hepatica* tegumental cell
WC(1)	-	Workshop Cluster (1)
WHO	-	World Health Organisation
Ym1	-	Yohimbine-1
$ZnSO_4$	-	Zinc Sulphate

Chapter 1

Introduction

1.1 General Introduction

In 2011 the global human population passed 7 billion. Conservative estimates predict this figure will rise to over 9.5 billion by 2050 (United Nations, 2013). Whilst the progressive mechanisation and intensification of food production systems and increases in global trade have satisfied demand to date, there is growing concern that further increases will be insufficient to support projected population growth due to limited availability of land and water (Suweis et al., 2015). There is a need therefore to use the resources already available in a more efficient and sustainable manner (Godfray et al., 2010). Current figures estimate that approximately one third (1.3 billion tons) of food produced globally is wasted through inefficiency at all levels of the food supply chain (FAO, 2011). Reducing such overt wastage should therefore be considered an imperative.

Animal based production systems will continue to play an important role in future global food supply; changing dietary habits, particularly in the developing world, will necessitate a two-fold increase in meat production by 2050 compared with only a 1.5-fold increase for cereal crops (Alexandratos and Bruinsma, 2012). If used appropriately, livestock can be used to increase overall food production efficiency through the use of what would otherwise be waste products as feedstuffs in intensive production systems, whilst extensive grass and forage based systems allow access to land that would otherwise be unavailable (Fitzpatrick, 2013; Godfray et al., 2010).

In this context, dairy farming is of particular note as a predominantly grass based system capable of achieving energy and protein conversion efficiencies comparable to those of more intensive cereal based production systems (Wedin et al., 1975). As a result of selective breeding, improved animal husbandry and nutrition, milk yields have increased dramatically over the course of the last century with average UK milk yields rising from 2000 to over 6000 litres per cow lactation between 1935 and 1995 (Simm, 2000). Recent data states the average modern UK Holstein-Friesian is capable of producing over 9000 litres per lactation (CDI, 2013). There are, however, drawbacks to grass based animal production systems. Greenhouse gas emissions are cited as a major issue from the perspective of environmental sustainability, with beef and dairy production being the two largest contributors to this issue in the livestock production sector (Lesschen et al., 2011). Consequently, factors which limit the efficiencies of these systems are important from the perspective of both food production and environmental impact. Worldwide, roughly 10-20% of meat production losses occur during the early stages of

the food supply chain which includes farm level production, slaughter and processing, whilst 3-4% of total milk losses occur at the farm level (FAO, 2011).

Veterinary infectious diseases represent a major source production loss and are also important due to their negative impact on animal welfare, which aside from being an important ethical consideration can further impair productivity (Fraser et al., 2013). Furthermore, many of these infectious diseases also have wider implications as zoonoses, making their control important not only for food security, but also from an animal health, welfare and public health standpoint.

1.2 The Liver Fluke *Fasciola hepatica*

The digenean trematode *Fasciola hepatica* is a parasite capable of infecting a wide range of vertebrate host species, including humans (Boray, 1969). With a worldwide distribution and a propensity for infecting livestock, particularly sheep and cattle, it is a parasite of major importance in terms of global food security and animal health and welfare, whilst its status as a zoonosis also makes it an important public health issue in many parts of the developing world (WHO, 2010).

1.2.1 Life Cycle

Fasciola hepatica has a complex life cycle with multiple developmental stages. *F. hepatica* eggs are shed by adult parasites residing within the hepatic bile ducts of their definitive host and passed out in the faeces onto pasture. Under the correct environmental conditions these undergo embryonation to produce miracidia, the transient free living stages with the sole purpose of seeking out and infecting an appropriate intermediate host. Embryonation is dependent upon environmental conditions, with ambient temperature a key determinant of the rate of development. A temperature range of 18-23^{o}C has been shown to be optimal for egg survival and development, with embryonation taking 15-25 days (Rowcliffe and Ollerenshaw, 1960). Outside this range efficiency is reduced; at lower temperatures development is slowed considerably, whilst with increasing temperatures egg mortality increases; below 9.5^{o}C and above 37^{o}C egg development does not occur (Rowcliffe and Ollerenshaw, 1960). In addition to temperature, several other factors are known to be important with respect to egg development including a pH range of 4.2-9.0 and sufficient levels of moisture and oxygen tension (pO_2). The conditions found within faecal material are not supportive of embryonation, and consequently egg development does not occur until they have been liberated into the wider environment (Rowcliffe and Ollerenshaw, 1960).

Like many other parasitic trematodes, *F. hepatica* requires a molluscan intermediate host as part of its life cycle. The mud snail *Galba truncatula* is the predominant intermediate host of *F.*

hepatica globally, although other species of lymnaeid snails are susceptible to infection (Bargues et al., 2011; Bargues and Mas-Coma, 2005; Mas-Coma et al., 2001). The life span of the miracidium is short due to its high energy consumption and limited glycogen reserves, only surviving in the environment for a period of hours (Graczyk and Fried, 1999). Embryonated eggs only hatch under a specific set of environmental conditions that must include a strong light stimulus and high levels of moisture. Once hatched, the miracidium swims using its cilia, guided by a number of mechanisms including photo- and chemotaxis in order to locate and infect an intermediate host (Andrews, 1999). If an appropriate snail candidate is located, the miracidium will attempt to penetrate it's body wall through a combination of mechanical boring and secretion of proteolytic enzymes (Smyth and Halton, 1983).

Once within its intermediate host the miracidium undergoes a number of metamorphoses, developing first into a sporocyst, then rediae, of which there may be multiple generations before giving rise to cercariae which are shed from the snail to form the infective stage, metacercariae (Andrews, 1999). The main purpose of the redial stages is to facilitate clonal expansion; a snail infected with a single miracidium can go on to produce hundreds or even thousands of cercariae over the course of weeks and months. Snail infections are generally life-long, with shedding typically beginning 4-7 weeks post infection and may occur intermittently multiple times throughout the snail's life (Andrews, 1999; Boray, 1969; Lee et al., 1995). These cercarial sheds occur under conditions which coincide with optimal snail activity (Graczyk and Fried, 1999). Cercariae, like miracidia, are a motile free living stage. They possess a tail which propels them through the environment, guided by positive phototaxis and negative geotaxis until they encounter a solid object, which under natural conditions is usually grass or similar herbage (Ginetsinkaya, 1988). Here they encyst to become metacercariae, shedding their tails and secreting a mixture of proteins and mucopolysaccharides to adhere to the plant surface (Dixon, 1965). These infective cysts can remain viable in the environment for several months, up to and in excess of a year as a result of the secreted outer cyst wall making them resistant to sub-zero temperatures and desiccation, whilst some secreted components inhibit fungal and bacterial contamination also (Andrews, 1999; Dixon, 1965).

Infection of the definitive host occurs through the ingestion of encysted metacercariae present on forage. Within hours of ingestion, the increase in extrinsic temperature combined with changes in pH and pCO_2 induces excystation, which ultimately occurs at the junction of the common bile duct and duodenum in the presence of bile salts (Dixon, 1966; Fried, 1994). These newly excysted juvenile fluke (NEJs) penetrate the wall of the small intestine to gain access to the peritoneum which they then migrate across to reach the liver, penetrating the capsule and migrating through the hepatic parenchyma to finally gain access to the bile ducts (Andrews, 1999). Throughout this migratory phase the juvenile fluke continually grow and

develop, particularly during the hepatic stages when they feed on blood and tissue (Boray, 1969). By the time they reach the bile ducts from 7-8 weeks post infection onwards, the parasites have grown to an adult size. Sexual maturity is achieved shortly after this, with a typical pre-patent period in sheep and cattle quoted as 10-12 weeks (Dixon, 1964; Ross et al., 1966). Whilst adult fluke are hermaphrodites and capable of parthogeny, adult parasites are known to cross-fertilize when multiple fluke are present within an animal (Vazquez et al., 2016). Egg production is prolific, with a single adult capable of producing up to 20,000 eggs per day, although this figure can vary greatly as a consequence of a number of different factors including animal age, infection intensity and duration, circadian rhythm and time of year (Boray, 1969; Gonzalez-Lanza et al., 1989; Honer, 1965a, b). Eggs are passed down the biliary tree to the bile duct and gall bladder before being passed into the duodenum where they mix with digesta and are ultimately passed in the faeces, thus completing the life cycle.

1.2.2 Epidemiology

It is apparent from the life-cycle (Section 1.2.1) that speed and prolificacy of *F. hepatica* development is largely governed by environmental factors, particularly moisture and ambient temperature. These are important for both the development of *F. hepatica* itself and its intermediate snail host. *F. hepatica* is found globally in both temperate and tropical regions. Differences exist in terms of development and transmission between these two climatic regions; parasite and snail development are generally limited by temperature in temperate regions, whilst in tropical climates moisture is generally the more important limiting factor (Torgerson and Claxton, 1999). In both cases, environmental development and transmission follows a seasonal pattern, with maximal development occurring during periods of warm, wet weather. Under optimal conditions, development from ovum to metacercariae can be as short as 35 days, meaning that in the weeks and months following such conditions pastures may become contaminated with a large infectious burden (Torgerson and Claxton, 1999). Under sub-optimal conditions, infectious burdens can still accumulate on the pasture through prolonged snail infection and survival of metacercarie on the pasture (Andrews, 1999). Furthermore, both metacercariae and infected snails are capable of surviving sub-zero temperatures, providing a mechanism through which the parasite can survive over winter in temperate regions where freezing conditions are common (Taylor et al., 2016).

In the UK, the close relationship between temperature, moisture and *F. hepatica* development have allowed the construction of a mathematical model which uses climatic data in the form of mean daily temperature and evapotranspiration as an indicator of moisture levels to predict periods of risk for high disease transmission, with demonstrable accuracy over the past 50 years (Fox et al., 2011; NADIS, 2016; Ollerenshaw, 1966). When these models are used to

forecast future disease risks in the UK and Europe following the projected trends in climate change, they predict that *F. hepatica* will become much more widely distributed in the coming decades both spatially and temporally (Caminade et al., 2015; Fox et al., 2011), with evidence to suggest that such effects are already being observed in many parts of Europe (Daniel et al., 2012; McCann et al., 2010; Olsen et al., 2015; Salimi-Bejestani et al., 2005a; Sekiya et al., 2013).

In addition to environmental factors, presence of the snail intermediate host is clearly essential. This is dependent upon a number of factors in addition to temperature and moisture such as soil pH, food availability etc. (Torgerson and Claxton, 1999). *Galba truncatula* are typically found residing in wetland environments such as shallow pools, ditches, marshy grassland, bogs and wet flushes on hillsides and are also able to colonise temporary water bodies and tolerate trampling by livestock and muddy and polluted water conditions (Kerney, 1999; Seddon et al., 2015), making them well suited to living in close proximity with the major definitive host species of *F. hepatica*. Adult snails may live for 12-14 months, potentially producing several thousand eggs during this time, which under optimal conditions can develop into sexually mature adults in 3-4 weeks (Kendall, 1953; Over, 1982; Torgerson and Claxton, 1999). This reproductive potential has implications for *F. hepatica*, as the prevalence of its intermediate host in the environment ultimately affects disease transmission. Furthermore, similarities exist between the optimal developmental ranges of *G. truncatula* and the environmental stages of *F. hepatica; G. truncatula* exhibits maximal growth and reproduction in an ambient temperature range of 18-27^{o}C (no development occurs below 10^{o}C) in pH range of 6.0-8.6 (Kendall, 1953; Over, 1982; Walton and Wright, 1926). Whilst *Galba* spp. are the predominant intermediate host species globally there are other *Lymnaeid* snail species capable of supporting *F. hepatica* infection and development which may alter epidemiology and parasite range in some areas, such as *Radix* spp. (Howell et al., 2012; Relf et al., 2009). With respect to the definitive host, most transmission occurs within and between livestock species, with purchase and introduction of infected animals being an important factor in governing spread of both disease and anthelmintic resistance (Howell et al., 2015; Soares et al., 2007; Williams et al., 2014) , whilst some sylvatic species may be important in acting as reservoir hosts (Section 1.2.4).

An additional important feature of *F. hepatica* in terms of its overall impact on food security is its relationship with co-infecting pathogens. This includes evidence of *F. hepatica* infected cattle being persistent faecal carriers of the zoonotic pathogen *Salmonella dublin* following experimental infection (Aitken et al., 1979), whilst an epidemiological association has been demonstrated in the Netherlands (Vaessen et al., 1998). More recently, cattle co-infected with *Mycobacterium bovis*, the causative agent of bovine tuberculosis, and *F. hepatica* have been

shown to exhibit a reduced response to the single intradermal comparative cervical tuberculin (SICCT) test commonly used for the diagnosis of *M. bovis* infection in UK cattle when compared to those infected with *M. bovis* alone (Flynn et al., 2007b; Flynn et al., 2009). Furthermore, (Claridge et al., 2012) demonstrated this relationship in the UK through epidemiological modelling, leading to the conclusion that co-infection with *F. hepatica* causes under diagnosis of *M. bovis* nationally. Bovine tuberculosis is a disease of major global importance in terms of both animal and human health. Current procedures to control and eradicate *M. bovis* from UK cattle have cost an estimated £500m over the past decade (DEFRA, 2014). The potential involvement of *F. hepatica* in impeding the control of this economically important zoonosis further emphasises its importance in global food security.

1.2.3 Disease and Pathogenesis

The migrating juvenile and adult stages within the definitive host cause a number of pathological changes, which if severe lead to overt clinical signs and disease (fasciolosis). The initial migration of NEJs through the small intestinal wall is achieved through the secretion of proteolytic enzymes (Behm and Sangster, 1999). Although this action necessitates some tissue damage, the evidence of this activity is difficult to detect post mortem and consequently poorly defined, although some increased infiltration by eosinophils is detected in the mucosa of experimentally infected rats (Van Milligen et al., 1998). Intestinal migration is not associated with overt clinical signs, even following heavy infection, unlike other parasitic trematodes such as Paramphistome spp. rumen fluke (Millar et al., 2012). Similarly, migration of the parasite through the peritoneum is not associated with overt clinical signs or pathological changes.

It is only when the juvenile fluke reach the liver that significant pathological changes and clinical signs are seen. Penetration of the liver capsule and subsequent tissue damage in heavy infections with several thousand fluke may result in lethal haemorrhage and sudden death with no prior indication of illness (Behm and Sangster, 1999). This scenario, referred to as acute type-1 fasciolosis is relatively rare in cattle. More usually, disease progresses as juvenile fluke migrate through the hepatic parenchyma, forming migratory tracks and significant tissue damage in the process. Livers become congested and enlarged, with parasitic tracks easily visible as gross pathological lesions from 5-6 weeks post infection (Ross et al., 1966). The majority of these tracks appear on the visceral surface of the left (ventral) lobe, which lies adjacent to the duodenum (Behm and Sangster, 1999; Dyce et al., 2002; Ross, 1968a). As hepatic migration progresses, the regional hepatic lymph nodes become enlarged, with histopathology of these showing germinal centre proliferation of plasma B-cells (Rahko, 1969). Histopathological examination of liver tissue at this stage of infection shows presence of erythrocytes, fibrin and cellular debris within the lumen of the parasitic tracks and local

inflammatory cell infiltrates consisting predominantly of neutrophils, eosinophils and lymphocytes both in tracks themselves and the surrounding tissue, with further infiltrates of macrophages and epithelioid cells also seen in the adjacent tissues (Jones et al., 1997; Meeusen et al., 1995). Whilst inflammatory cells are present within the parasitic tracks, it has been noted that these are present in greatest numbers in the regions behind the migrating juveniles rather than surrounding the parasites themselves (Meeusen et al., 1995; Zafra et al., 2013). These pathological changes may result in clinical disease commonly referred to as acute type-2, or subacute fasciolosis associated with heavy infectious burdens with signs including anaemia, loss of body condition and a general ill thrift which may manifest as production losses and poor coat condition or wool break in sheep (Behm and Sangster, 1999; Taylor et al., 2016). Assessment of clinical parameters may also reveal hypoproteinaemia resulting from blood loss and impaired liver function, eosinophilia and elevations of the liver enzyme glutamate dehydrogenase (GLDH) in serum as a result of hepatocellular damage caused by the migrating fluke (Taylor et al., 2016). Gammaglobulinaemia is also usually observed at this stage, and is of particular interest from a diagnostic perspective (Anderson et al., 1977; Salimi-Bejestani et al., 2005b) (Section 1.2.6).

As juvenile fluke complete their migration to the hepatic bile ducts, the liver tissue becomes cirrhotic as old tracks heal through fibrosis with fibrinous deposits seen over the liver capsule. The bile ducts become inflamed as a result of adult fluke causing damage to the biliary tract epithelium as they move using their tegumental spines. Additionally, in cattle this can lead to calcification of the bile ducts, colloquially referred to as ‘pipe-stem liver’ (Jones et al., 1997; Ross, 1968a; Taylor et al., 2016). On histopathology, old migratory tracks show extensive fibrosis and healing through granulation, with eosinophils and neutrophils being replaced by macrophage infiltration, whilst bile ducts show epithelial hyperplasia and fibrosis (Jones et al., 1997; Rahko, 1969). In these chronic stages of infection a regenerative anaemia is usually present, maintained through active blood feeding by the adult parasites (Symons and Boray, 1967), whilst hypoalbuminaemia, eosinophilia, gammablobulinaemia and elevations in serum gamma-glutamyltranspeptidase (GGT) (resulting from damage to the biliary epithelium) are all common clinical features. Clinical fasciolosis in the chronic stages results from low infectious burdens of up to several hundred fluke, although such infections are often sub-clinical. If, however, overt chronic disease is present, clinical signs include pallor and anaemia, ill thrift and poor body condition, whilst impaired liver function and chronic hypoproteinaemia can result in the classical sign of submandibular oedema or ‘bottlejaw’ (Taylor et al., 2016). An additional and important sequelae of *F. hepatica* infection in the chronic stages of disease is that of secondary infection with *Clostridium novyi*, causing ‘black disease’ (Bagadi and Sewell, 1973; Kelch et al., 1977). The presence of necrotic tissue left in the wake of fluke migration

provides optimal conditions for colonisation by the bacterium, which usually leads to sudden death.

1.2.4 Definitive Host Species

Fasciola hepatica differs from the most other parasitic helminths, being capable of infecting a wide range of vertebrate hosts. In addition to sheep and cattle, several other domesticated species are known to be susceptible, including new world camelids (Duff et al., 1999; Puente, 1997), goats, equids and pigs, although there is a marked difference in host susceptibility between these (Boray, 1969). There is also evidence of grazing avian livestock such as Rheas becoming infected under natural conditions (Soares et al., 2007), whilst a number of wild species also act as definitive hosts including hares, coypus, deer and wild boar in Europe (Arias et al., 2013; Menard et al., 2001; Mezo et al., 2013; Walker et al., 2011), camelids, hares and coypus in South America (Cuervo et al., 2015; Issia et al., 2009) and marsupial species, particularly possum, in the antipodes (Boray, 1969).

Fasciola hepatica is also a major zoonosis in many parts of the world. As with other species, infection is through consumption of infective cysts on herbage, although disease carrying domestic and wild species are important in terms of the epidemiology of human fasciolosis (Mas-Coma et al., 2009). Disease and pathogenesis is similar in people to that previously described, with the additional symptom of abdominal pain (Arjona et al., 1995). Further complicating the issue of human fasciolosis is the fact that it may mimic other hepatopathies, particularly neoplasia, resulting in misdiagnosis and treatment (Losada et al., 2015; Yalav et al., 2012). It is thought that at least 2.6 million people are infected globally at a cost of around 35,000 DALYs (Furst et al., 2012).

1.2.5 Impact on Food Security

Fasciolosis in livestock has a significant impact on food security and economics. Where meat hygiene inspection is implemented, it is common policy to condemn livers showing signs of infection with *F. hepatica* as unfit for human or animal consumption as a result of spoilage predominantly, although potential zoonotic transmission through consumption of infected livers has also been demonstrated (Taira et al., 1997). In the UK, abattoir statistics from 2010 showed over 800,000 sheep livers and over 500,000 cattle livers were condemned as a result of *F. hepatica* infection at a combined cost of approximately £3 million (EBLEX, 2011). In addition to this direct loss from the food chain, *F. hepatica* exerts a number of additional effects on its definitive host which ultimately impair its productivity. In sheep, mortality caused by acute (type-1) fasciolosis (and secondary clostridial disease) can occur in a significant proportion of flocks grazing high risk pasture (Bosco et al., 2015) whilst in peracute (type-2)

chronic and sub-clinical disease, the resulting anaemia and hepatic damage can cause significant reductions in weight gain and food conversion efficiency in growing lambs (Sykes et al., 1980), whilst in ewes it negatively affects fertility, body condition, milk production, wool growth and fleece quality in addition to increasing their susceptibility to metabolic diseases such as pregnancy toxaemia (Sargison and Scott, 2011).

In cattle, clinical disease is much less common. Sub-clinical chronic infections may go undiagnosed and therefore have ongoing negative effects on productivity (Andrews, 1999). Chronic disease in cattle has been shown to reduce weight gain and delay sexual maturation in youngstock (Charlier et al., 2007; Lopez-Diaz et al., 1998), whilst in adult dairy cattle a number of studies have demonstrated a negative relationship between fasciolosis and milk yields, with figures suggesting that presence of disease within a lactating herd can result in an 8-15% reduction in yields, whilst reductions in butterfat content have also been reported (Charlier et al., 2007; Howell et al., 2015; Kuerpick et al., 2012; Mezo et al., 2011; Ross, 1970; Schweizer et al., 2005).

In addition to production based effects, further costs are incurred through treatment regimens with anthelmintics, whilst several studies in sheep and cattle have shown that *F. hepatica* infection interferes with the liver's ability to metabolise a number of pharmacological agents, including antimicrobials, corticosteroids and barbiturates which has several implications for food safety (Behm and Sangster, 1999). Furthermore, its impact on host responsiveness and susceptibility to other production limiting and zoonotic diseases raises additional issues over food security from the perspective of hygiene and safety (Aitken et al., 1979; Claridge et al., 2012; Vaessen et al., 1998)

Due to the myriad of contributing factors it is difficult to quantify the overall impact of *F. hepatica* on food production and security. Estimates have been made as to the global impact of *F. hepatica* which suggest an economic cost in excess of $2bn per anum (Spithill et al., 1999), with costs to the UK economy resulting from production losses, abattoir condemnation and cost of treatment estimated to be £13-15 million in the sheep industry (£25-30 per animal) (EBLEX, 2011) and £23 million in the UK cattle industry, or £89 per beef cow and £162-224 per dairy cow (Bennet and Ijpelaar, 2003; EBLEX, 2015). Whilst these figures are historical and do not account for all potential economic impacts they do emphasise the importance of *F. hepatica* to the global and UK economy.

1.2.6 Diagnosis

Whilst history and clinical findings are often indicative of *F. hepatica* infection, use of specific tests improves diagnostic accuracy. There are a number of tests available, each with relative merits and drawbacks.

Faecal egg counts are the most widely used, with a variety of methods described for refining and separating *F. hepatica* eggs from faecal material. Due to the high density of *F. hepatica* eggs sedimentation methods are commonly used, although flotation can be achieved using a solution with a high specific gravity such as zinc sulphate (Taylor et al., 2016). Both sedimentation and flotation techniques involve initial sieving of the sample, after which flotation techniques require centrifugation and re-suspension of sample in saturated $ZnSO_4$ or similar media prior to loading into a counting chamber similar to those used for nematode eggs for microscopic examination (Charlier et al., 2008a; Cringoli et al., 2010; Taylor et al., 2016). With sedimentation, after sieving a series of re-suspensions and sedimentations in water are required to remove debris; refinements have been attempted to improve egg recovery in this process through the use of detergents, whilst techniques that add a flotation step following sedimentation have also been described (Charlier et al., 2008a; Conceicao et al., 2002; Happich and Boray, 1969; Suhardono et al., 2006). There is little evidence, however, that these various refinements have any significant impact on improving the basic sedimentation method. The major determining factor in egg count sensitivity appears to be the quantity of faeces available for processing in the first instance; Rapsch et al. (2006) found test sensitivities of 69% when 10g of faeces were used, increasing to 91.9% when 30g of faeces was processed. In this respect, sedimentation is the better of the two techniques, as it permits larger quantities of faeces to be analysed (Happich and Boray, 1969).

F. hepatica eggs are large (130-145 x 70-90µm) compared with those of other common parasitic helminths, whilst their golden brown colouration and refractile appearance makes them easily identifiable amongst sample detritus, although this can be further enhanced through the use of methylene blue as a background stain. Microscopic examination can be performed under relatively low power magnification (4-10x) enabling the large quantities of faecal material required to be examined. Irrespective of the techniques used, diagnosis through the demonstration of faecal eggs is only capable of detecting patent infections. Furthermore, whilst highly specific (at or close to 100%) (Charlier et al., 2008a; Rapsch et al., 2006), it is known that eggs can be retained within the gall bladder of infected individuals for a period of several weeks, in some cases up to 16 weeks post treatment, meaning treatment history must be taken into account when interpreting results (Toner et al., 2011). Fluke egg counts are mainly used qualitatively; although there is some correlation with burden the relationship is not sufficiently close to be informative, possibly as a result of eggs production per fluke being reduced in heavier infectious burdens (Boray, 1969). Egg sedimentations are laborious to process and examine, taking several hours per sample to complete, although analysis of composite samples have been shown to be as effective as individual egg counts in sheep for diagnosis at the group level, expediting the process somewhat (Daniel et al., 2012).

Enzyme-Linked Immunosorbent Assays (ELISAs) have been developed for the detection of *F. hepatica* specific IgG antibody in serum samples from sheep, cattle, horses and humans (Caban-Hernandez et al., 2014; LVPD, 2015; Reichel, 2002; Salimi-Bejestani et al., 2005b). These are capable of detecting infection from as early as 2 weeks, with a quoted sensitivity of 98% and specificity of 96% at the standardised diagnostic cut-off for an anti-E/S antigen ELISA (Salimi-Bejestani et al., 2005b). This allows for the rapid diagnosis of multiple samples simultaneously, whilst in dairy cattle there is the added benefit of being able to use milk samples, making sample collection straightforward and also enabling the ELISA to be used as a screening test for groups of lactating animals by analysing composite bulk tank samples (Salimi-Bejestani et al., 2005a; Salimi-Bejestani et al., 2005b). Whilst antibody ELISAs have an increased sensitivity over worm egg counts, these detect host response rather than infection directly. Consequently, issues may exist surrounding test specificity, particularly in terms of historical infections following treatment or spontaneous resolution of disease. There is also some variation in the relative sensitivity and specificity of different ELISAs depending on the antigens used to detect host IgG at different times of year. This appears to be related to stage of infection and seasonality of the disease altering the degree of host exposure to the respective antigen fraction at these different disease stages (Charlier et al., 2008a; Kuerpick et al., 2013).

A commercial copro-antigen ELISA also exists which detects *F. hepatica* antigen in faeces. Whilst this test has been shown to be sensitive at detecting infection from 5 weeks post infection onwards in experimental infection (Mezo et al., 2004), recent studies examining natural infection have suggested a lower sensitivity than previously thought, with quoted sensitivities more akin to those given for faecal egg counts (Duscher et al., 2011).

In addition to these three main diagnostic tests, others also exist but aren't currently widely used, including DNA based techniques like Polymerase Chain Reaction (PCR) and Loop Mediated Isothermal Amplification (LAMP) assays which have been used in the diagnosis of *F. hepatica* infection in cattle (Ai et al., 2010; Martinez-Perez et al., 2012) and may also have potential uses for the detection of *F. hepatica* in the environment, particularly to identify infected snail populations (Ai et al., 2010; Alba et al., 2015). Lateral flow technology has been developed in humans to detect *F. hepatica* antibodies which due to its portable nature may have potential use in the veterinary field also (Martinez-Sernandez et al., 2011).

1.2.7 Treatment and Control

The current methods employed to control *F. hepatica* in livestock are based predominantly on the use of a relatively small number of anthelmintics with efficacy against *F. hepatica*. These 'flukicides' are all effective at killing adult fluke, but have varying degrees of efficacy against

the earlier juvenile stages; triclabendazole (TCBZ) has a high level of efficacy against both adult and juvenile fluke down to 2-3 days of age (Boray et al., 1983), whilst closantel and nitroxynil have limited efficacy (50-90%) against juvenile stages from 7-9 weeks onwards, with clorsulon effective in killing juvenile fluke from 8 weeks post infection when administered orally. Rafoxanide (currently not licenced for use in the UK) has a 50-90% efficacy against juvenile fluke between 3-6 weeks post infection (Fairweather and Boray, 1999), whilst albendazole and oxyclozanide are only effective against adult parasites from 12 weeks post infection. A summary of products licenced for use in cattle in the UK is shown in table 1.1. None of these flukicides have residual activity and are only effective at the time of administration. Conversely, these products have long drug withdrawal periods, which is particularly problematic in dairy cattle. In the UK the majority of products are not licenced for use in animals producing milk for human consumption, with only albendazole and oxyclozanide licenced for use during lactation. The high efficacy of TCBZ in killing juvenile fluke makes it the only realistic choice for treating acute disease. As a consequence, since its introduction in the 1980s it has become the most commonly and widely used of these products. Due to this reliance, particularly in sheep TCBZ resistant fluke populations have begun to emerge in many parts of the world, with reports becoming increasingly common in regions with high disease prevalence (Brockwell et al., 2014; Gordon et al., 2012; Mitchell et al., 1998; Moll et al., 2000; Olaechea et al., 2011; Ortiz et al., 2013; Overend and Bowen, 1995; Thomas et al., 2000), Resistance to other products like closantel and rafoxanide has been demonstrated experimentally, with recent reports suggesting the emergence of resistance to closantel in cattle in the field (Fairweather and Boray, 1999; Novobilsky and Hoglund, 2015).

Whilst the exact mechanism of action of TCBZ in killing *F. hepatica* is unknown, the mechanisms of drug resistance are better understood. These are related to increased drug metabolism by the parasite to a less pharmacologically active form, a reduced uptake and increased efflux (Fairweather, 2011). It has been shown that some of these resistance mechanisms, particularly P-glycoprotein (Pgp)-linked drug efflux can be reversed through co-administration of TCBZ with a Pgp-inhibitor such as ivermectin or verapamil *in vitro* (Mottier et al., 2006; Savage et al., 2013), although to date these effects have not been replicated *in vivo* (Ceballos et al., 2010). Resistance traits are heritable, with resistance gene alleles increasing in frequency within wild-type populations as a result of selection pressures resulting from prolonged and repeated use of TCBZ, as has been demonstrated with resistance of other helminth and protozoal parasites (Cui et al., 2015; Taylor, 2012). Through genomic analysis, efforts are being made to better understand the genetic basis of drug resistance by comparison of susceptible and resistant fluke populations with a view to uncovering novel ways of counteracting these traits (Hodgkinson et al., 2013).

There are currently no new drugs in development, meaning it is likely TCBZ will continue to be a central feature of disease control for years to come. It is vitally important therefore that its use is limited where possible to preserve efficacy. In the UK, initiatives have been established to promote the responsible use of anthelmintics (including TCBZ) to achieve exactly this goal (COWS, 2013; SCOPS, 2012). These promote the use of alternative control strategies in conjunction with anthelmintic treatment, which in the case of *F. hepatica* include reducing transmission potential through either fencing off areas where disease transmission is thought/known to occur, or avoiding grazing these areas during high risk periods as predicted by parasite forecasting systems as previously described (NADIS, 2016) (Section 1.2.2). Where flukicides are used, a greater emphasis should be placed on diagnostics, to avoid misuse and/or inappropriate treatments being administered, with rotational use of alternative products during periods when acute fasciolosis is unlikely, for example the use of oxyclozanide or similar adult flukicide in animals that have been housed for a period of several weeks, and use of alternative products as quarantine drenches for farmers without TCBZ resistance when buying in livestock (Sargison, 2006). Although co-grazing with sheep is beneficial for control of some other parasitic helminth species, it has been shown that co-grazing sheep and cattle increases disease, and may provide additional routes for bringing resistant parasites onto farms, such as when hill sheep are overwintered on lowland pasture grazed by cattle in the summer months (COWS, 2013). Threshold treatments based on cost efficiency have been proposed, with animals only treated when clinical parameters reach a certain level, with egg counts >5 eggs per gram, GGT serum levels >150u/l or a herd prevalence >25% all proposed as therapeutic cut-offs (Vercruysse and Claerebout, 2001). However, with a disease like fasciolosis that has a life cycle with massive potential for proliferation in the environment it is difficult to see how such theory could be put into practice without risking major disease breakdowns in the future seasons.

Control of snail populations has also been proposed previously; at one time pasture treatment with molluscicides was looked upon favourably, but has subsequently been abandoned in the UK due to the detrimental effects such practices have on the environment (Torgerson and Claxton, 1999). Drainage of marshland can be used to reduce snail habitat ranges, whilst alternative methods to control of both snails and environmental stages of *F. hepatica* have been described including the use of certain species such as water fowl to predate lymnaeid snails (Levine, 1970), and infection of *G. truncatula* with alternative helminth species which compete with and antagonise *F. hepatica* development (Torgerson and Claxton, 1999).

Table 1.1: Flukicides licenced for use in cattle in the UK

Stage	Juvenile efficacy	Adult efficacy	Drug	Trade names (route of administration)	Milk Withdrawal	Meat Withdrawal
Adult	50-70% at 10-11 weeks	80-99% effective from 12 weeks	Albendazole	Albex 2.5% (drench)	60 hours	14 days
				Albex 10% (drench)		
				Albenil 2.5% (drench)		
				Albenil 10% (drench)		
				Endospec 2.5% (drench)		
				Endospec 10% (drench)		
				Ovispec 2.5% (drench)		
				Ovispec 10% (drench)		
			Oxyclozanide	Zanil (drench)	72 hours	28 days
				Douvistome (drench)[1]	nil	14 days
			Clorsulon	Animec super (injectible)	Not licensed for use within 60 days of calving	66 days
				Ivomec super (injectible)		
				Virbamec super (injectible)		
Intermediate	50-90% at 7-9 weeks	91-99% effective from 10 weeks	Nitroxynil	Trodax 34% (injectible)	Not licensed	60 days
			Closantel	Closantel 20% (pour-on)	Not licensed for use within 60 days of calving	28 days
				Closantel 12.5% (pour-on)		49 days
			Triclabendazole (pour-on)	Cydectin Triclamox (pour-on)	Not licensed	143 days
Juvenile	90-99% at 1-3 weeks	99-99.9% effective from 4 weeks	Triclabendazole	Combinex (drench)	Not licensed	56 days
				Tribex 10% (drench)		
				Fasinex 10% (drench)		
				Fasinex 100 10% (drench)		
				Endofluke (drench)	Not licensed for use within 45 days of calving[3] Milk must be discarded for first 48 hours of lactation post calving	56 days
				Fasinex 240 24% (drench)	Not licensed for use within 48 days of calving[2] Milk must be discarded for first 48 hours of lactation post calving	52 days

1. Not a UK licensed product, obtainable through import from Europe.
2. For cattle treated within 45 days of calving, milk must be discarded until 47 days (45 days plus 48 hours) after the date of treatment.
3. Treated within 48 days of calving, milk must be discarded until 50 days (48 days plus 48 hours) after the date of treatment.

1.3 Immunology

Understanding the bovine immune response to *F. hepatica* infection is important due to the potential effects these have on *F. hepatica* infection itself, particularly in the context of vaccine development, and also with respect to the impact these may have on the immune responses to co-infecting pathogens. In the case of the vaccines, identifying which immune responses are capable of eliminating infection and those which are non-protective will help to inform their development in terms of antigen choice, adjuvant and route of administration.

1.3.1 The Mammalian Immune System

Early immune responses are initiated by innate sentinel cells, principally macrophages (Mϕ), mast cells (MCs) and dendritic cells (DCs) residing within extravascular tissues, monitoring the environment for pathogen-associated molecular patterns (PAMPs) and/or endogenous alarmin molecules produced by damaged or distressed tissue cells. Sentinel cells identify PAMPs through a variety of pattern recognition receptors (PRRs) expressed on their cell surface including toll-like receptors (TLRs) and C-type lectins. These PRRs are capable of recognising a wide range of exogenous pathogen derived proteins, lipoproteins, carbohydrates and lipopolysaccharides (LPS) (Tizard, 2013). If any such material is recognised, sentinel cells become activated, usually eliciting a pro-inflammatory response. In the case of MCs this results in degranulation and release of pro-inflammatory cytokines and chemokines to recruit other innate immune cells to the region and histamine to increase vascular permeability to facilitate extravasation of leukocytes (Murphy et al., 2012). DCs and macrophages typically become activated in a similar manner if PAMPs are detected, releasing pro-inflammatory cytokines tumour necrosis factor(TNF)-α, interleukin (IL)-1, and IL-6 to initiate local inflammatory responses and chemokines to recruit innate cells such as neutrophils, monocytes and eosinophils from peripheral circulation.

Both DCs and macrophages act as antigen presenting cells (APCs), and initiate the adaptive immune response through phagocytosis and processing of pathogens and their associated molecules into peptides which, following migration through lymphatic vessels to a peripheral draining lymph node, are expressed on the APC surface bound to major histocompatibility complex (MHC)-II and are presented to $CD3^+$ $CD4^+$ T-helper cells. Each unique T-cell clone possesses a novel antigen specific T-cell receptor (TCR). If a $CD4^+$ $CD3^+$ T-cell recognises an antigen, activation occurs. Although TCR-antigen recognition is essential, activation is also dependent upon co-stimulatory signalling through additional surface receptor interactions between APCs and $CD4^+$ T-cells, including CD40-CD154 and CD80/86-CD28 binding and the presence of certain cytokines, usually produced by the APC (Tizard, 2013). Activated $CD4^+$ T-cells proliferate in a clonal expansion of antigen-specific $CD4^+$ T-helper cells which act within

lymph nodes and lymphoid tissues to activate additional components of the adaptive immune response, namely $CD8^+$ T-cells and B-cells, and migrate via the vascular system to sites of inflammation and colonise other regional lymph nodes in order to enhance systemic antigen detection.

In addition to their role as initiators of the adaptive response and APCs, macrophages also function as part of the innate cellular response. Usually, activated macrophages exhibit 'classical' M1 activation phenotypically characterised by increased motility, phagocytosis and killing of pathogens through the production of oxidative free radicals such as nitric oxide (NO) and peroxynitrite ($OONO^-$) and production of early stage pro-inflammatory cytokines including TNF-α, IL-12 and IL-18 which help to prime the adaptive immune response. Phagocytic function can be enhanced by other components of the immune system such as antibody and/or complement through Fc and complement surface receptor binding.

In addition to M1 activation, macrophages may also become activated via an alternative pathway. These 'alternatively activated' M2 macrophages differ in phenotype from M1 cells, displaying reduced phagocytic behaviour and oxidative production, with a switch in nitrogen metabolism from inducible nitric oxide synthase (iNOS) to arginase enzyme activity in addition to increased MHC-II cell surface expression, IL-10 production and corresponding reduction in pro-inflammatory cytokines (Tizard, 2013). M2 cells are normally associated with wound healing, fibrosis and tissue remodelling, and are also a prominent feature of many helminth infections (Gordon, 2003).

The adaptive immune system acts principally to co-ordinate and augment the local innate immune response through both local and systemic effects. These mechanisms roughly fall into one of two types of response largely determined by the phenotype of the $CD4^+$ T-helper cells activated.

T-helper 1 (T_H1) cells are induced in the presence of IL-12, interferon(IFN)-γ and IL-18 during antigen presentation (Tizard, 2013). Activated T_H1 cells then in turn produce TNF-α, IL-2 and IFN-γ, all of which are principally associated with stimulation of cell mediated immune responses; TNF-α is an acute phase cytokine commonly associated with a pro-inflammatory responses, IL-2 is an important co-stimulatory molecule involved in the activation and proliferation of $CD8^+$ T-cells and B-cells in the lymph node and IFN-γ activates tissue macrophages and increases phagocytic activity. In cattle, IFN-γ and IL-2 also induce IgG2 class antibody production by B-cells which aids the phagocytic activity of macrophages in an antibody-dependent manner through IgG2 specific Fc receptors (Fcγ2R) (Estes et al., 1994; Tizard, 2013).

T-helper 2 (T_H2) cell activation occurs in response to antigen presentation and IL-4 and IL-13 production as well as an absence of IL-12 (Tizard, 2013). Activated T_H2 cells subsequently

producing IL-4 and IL-5 which predominantly act to stimulate B-cell activation, proliferation and antibody production, of which IgG1, IgA and IgE are the predominant isotypes in a type-2 response. Antibodies assist in pathogen killing through a variety of mechanisms including antibody-dependant cell mediated cytotoxicity (ADCC) and complement fixation. Within inflamed tissues, IL-4 also activates MCs and IL-5 activates and recruits eosinophils, whilst IL-10 antagonises T_H1 cell function and suppresses classical M1 macrophage activation, whilst IL-4 and IL-13 induce alternative M2 activation (Gordon, 2003; Tizard, 2013). Type-2 responses are commonly associated with extracellular pathogens, including parasitic GI helminths, against which such immune responses are protective (Tizard, 2013).

In addition to these two polarised T cell responses additional phenotypes exist including T_H0 cells which produce a mixture of type-1 and type-2 cytokines resulting in a mixed type inflammatory reaction as is often observed in the early stages of infection, whilst regulatory T-cells (Tregs) act to control the magnitude of pro-inflammatory and proliferative responses through production of transforming growth factor (TGF)-β and IL-10 predominantly. Such regulatory mechanisms are important in chronic inflammatory and autoimmune diseases, where sustained pro-inflammatory responses would otherwise result in severe tissue damage and pathology. In mice and humans, functional Tregs have been phenotypically identified as $CD4^+$ $CD25^+$ $FoxP3^+$ T-cells (Hori and Sakaguchi, 2004). In cattle, although $FoxP3^+$ expression and the presence of functional $CD4^+$ $CD25^+$ Tregs have been identified (Coussens et al., 2012; de Almeida et al., 2008; Hoek et al., 2009; Seo et al., 2009), $WC1^+$ γδT-cells have also been shown to function as regulatory cells (Chiodini and Davis, 1992; Coussens et al., 2012; Hoek et al., 2009). Since $WC1^+$ γδT-cells are known to constitute a much higher proportion of the total lymphocyte population in ruminants when compared with other species (Tizard, 2013), it is unclear which phenotype is the more important regulatory subset in ruminants, although both are known to induce their effects through TGF-β and IL-10 production (Coussens et al., 2012; Hoek et al., 2009).

1.3.2 *F. hepatica*-specific Immunology

There are a number of studies investigating the immune responses to *F. hepatica* in cattle, the majority of which are based on experimental infections which follow two types of model, namely single dose or trickle infections. Earlier studies tended to observe immune responses to a single oral challenge of metacercariae, with more recent studies using 'trickle' infection where the dose is given in smaller quantities over a period of days or weeks in order to better simulate the type of infectious challenge that would be experienced in a natural challenge (Bossaert et al., 2000a; Clery et al., 1995). Studies comparing the differences between these two infection models observed lower serum antibody titres and proliferative responses by *ex*

vivo peripheral blood mononuclear cells (PBMCs) in trickle infections compared to single dose infections of metacercariae (Bossaert et al., 2000a; Bossaert et al., 2000b). A similar difference was observed in terms of the magnitude of antibody response when comparing cattle experimentally infected with a single dose of metacercaraie with naturally infected animals (Oblitas, 1997).

Prominent immunological changes associated with *F. hepatica* infection include eosinophilia, leukocytosis and parasite specific gammaglobulinaemia (Bossaert et al., 2000a; Bossaert et al., 2000b; Roberts, 1968; Ross et al., 1966; Sykes et al., 1980). In naturally and experimentally infected cattle, this gammaglobulinaemia is predominantly an IgG class antibody response to *F. hepatica* excretory/secretory (E/S) products (Clery et al., 1996; Ortiz et al., 2000). Further evaluation of these E/S antigen specific serum IgG responses have determined that IgG1 is the predominant antibody isotype in both single dose and trickle infections, although IgG2 isotype was found to be present at lower yet detectable titres (Bossaert et al., 2000a; Clery et al., 1996; McCole et al., 1999). With respect to naturally infected animals, Oblitas (1997) demonstrated no appreciable difference between IgG1 and IgG2 titres, thus highlighting potential differences between the responses of experimentally and naturally infected animals. In single dose infected cattle antibody responses were detectable from 2-3 weeks post infection and remained elevated for the duration of infection (Ross et al., 1966; Salimi-Bejestani et al., 2005b).

In spite of these observed immune responses, it is apparent from both experimental and natural infections that these are not protective against infection. A study from Galicia, northwest Spain has shown prevalence increases with age (Gonzalez-Lanza et al., 1989), whilst experimental infections have demonstrated that infection derived from a single infectious challenge can be maintained within ruminants for several months and even years (Mulcahy et al., 1999; Ross, 1968b) during which time the host remains susceptible to re-infection, suggesting these immune responses are not fully protective against infection (Bossaert et al., 2000a; Clery et al., 1996). This is believed to be the result of parasite evasion and modulation of the host response.

1.3.2.1 Mixed Proliferative Responses in Early Stage Infection

In cattle, strong parasite specific proliferation and IL-2 production are observed in *ex vivo* peripheral blood mononuclear cell (PBMC) cultures when stimulated with whole fluke homogenate (LFH) at 2 weeks post infection following a single dose challenge with 1000 metacercariae (Oldham and Williams, 1985). Similar parasite specific proliferation responses have been observed in *ex vivo* PBMCs taken from trickle infected animals at 1-3 weeks post infection (Bossaert et al., 2000b; McCole et al., 1999), whilst Clery and Mulcahy (1998) also

showed a strong IFN-γ production in response to both adult and juvenile fluke antigen during this period of proliferation. McCole et al. (1999) observed that γδT-cell depleted PBMC cultures showed attenuated proliferative responses at 10 days post infection, whilst $CD4^+$ and $CD8^+$ T-cell depletion resulted in an attenuation of these proliferative responses from 14 to 35 days post infection indicating all three T-cell phenotypes were involved in proliferative responses. In addition to parasite-specific responses, a transient increase in proliferative responses by *ex vivo* PBMCs taken from trickle infected cattle in cultures stimulated with the non-specific mitogen concanavalin A at 2 weeks post infection (McCole et al., 1998a). In experimentally infected sheep an initial *ex vivo* PBMC proliferative response was observed at 2 weeks post infection in response to stimulation with both whole fluke homogenate (LFH) and concanavalin A, with these proliferative responses shown to be negatively correlated with fluke burdens recovered post-mortem (Hacariz et al., 2009). Conversely, in cattle when cumulative PBMC proliferation was considered, this was found to correlate positively with mature fluke burden by Clery et al. (1996), whilst a similar finding was found by McCole et al. (1999) in addition to a positive correlation between PBMC proliferation and serum GLDH levels, suggesting these proliferative responses do not contribute towards a protective immune response.

Little is known of the local immune responses in the early stages of infection, although mononuclear cells recovered from the hepatic lymph nodes of cattle infected with single dose challenge of 375-500 metacercariae demonstrated IL-2, IL-4 and IFN-γ production at 10 and 14 days post challenge in response to LFH, whilst parasite specific IgG1 antibody was identified in unstimulated cultures suggesting production *in vivo* (Hoyle and Taylor, 2003); colonic tissue recovered from experimentally infected cattle demonstrated significant increases in eosinophil density within the intestinal mucosa also (McCole et al., 1998b).

In the mouse model, there is also evidence demonstrating modulation and regulatory function by both innate and adaptive immune cells in the early stages of infection. $CD11c^+$ cells (DCs and tissue macrophages) isolated from the peritoneal cavity of experimentally infected animals showing increased IL-10 and TGF-β production and a decrease in MHC-II, CD40, CD80/CD86 surface receptor expression, whilst in the peritoneum increased numbers of $CD4^+$ T-cells were found to express FoxP3 surface receptor and produce IL-10, but not IL-4 or IFN-γ compared to uninfected controls (Walsh et al., 2009). Similarly, *ex vivo* stimulation of splenic as well as hepatic and mesenteric lymph node cells taken from *F. hepatica* infected animals showed increased IL-4 and IL-5 and reduced IL-2 and IFN-γ by 3 weeks post infection (O'Neill et al., 2000).

1.3.2.2 Type-2 Immune Responses in Chronic Infection

The chronic immune response is a dominant feature of *F. hepatica* infection. PBMC proliferation and cytokine responses observed in early infection are either absent or become greatly diminished as the disease progresses; *F. hepatica* specific proliferation, IL-2 and IFN-γ production *ex vivo* by PBMCs was absent in experimental infections from 5 weeks post infection (Clery and Mulcahy, 1998; Oldham and Williams, 1985). Bossaert et al. (2000b) observed proliferation throughout the 24 week study period, but that these responses were strongest in the first 5 weeks of infection. Similarly, Flynn and Mulcahy (2008a) observed significant reductions in parasite specific PBMC proliferation from 4 to 12 weeks post infection and a significant reduction in ConA stimulated proliferation at both 4 and 12 weeks post-infection compared to pre-infection levels. McCole et al. (1999) showed proliferative responses were absent from 8 weeks post infection whereas IgG1 plasma concentrations reached peak titres at 5 weeks post infection before declining from 7 weeks onwards. This lack of proliferation in the chronic stages of infection indicates either an anergic or regulatory immune response, although evidence of impaired proliferative responses to ConA suggest the latter is more likely. This is further supported by evidence from experimentally infected sheep, where a significant suppression of PBMC proliferation compared to pre-infection levels was observed in response to both ConA and phytohaemagglutinin (PHA) from 4 weeks post infection onwards (Zimmerman et al., 1983).

A similar pattern is observed with peripheral blood eosinophil counts, peaking in early infection before a gradual decline following single dose infection, or increasing initially to reach a plateau in the chronic stages following trickle infection (Bossaert et al., 2000b; Ross et al., 1966).

The predominantly IgG1 isotype antibody response and eosinophilia present in chronic infections suggest the immune response is a type-2 response at this stage. This is supported by a study where parasite specific clonal populations of $CD4^+$ T-cells isolated from peripheral blood of single dose infected cattle from 27 weeks post infection onwards were found to produce either IL-4 exclusively or in combination with IL-2 and IFN-γ; none of the 17 clones isolated were found to express IFN-γ in isolation (Brown et al., 1994).

There is evidence to suggest that the progression towards a non-proliferative type-2 response in the chronic stages of infection is the result of immune regulation/modulation; experimental infections have demonstrated both IL-10 and TGF-β production by PBMCs in response to *ex vivo* stimulation with ConA and LFH (Flynn and Mulcahy, 2008b). IL-10 production was observed in response to ConA at 4 weeks post infection, and to both ConA and LFH at 12 weeks post infection whilst parasite specific TGF-β production was observed to be increased at both 4 and 12 weeks post infection compared to ConA stimulated cultures, with increased

production observed in both cultures compared to pre-infection levels. This study also demonstrated that following *in vitro* neutralisation of TGF-β both *F. hepatica* specific IL-4 and ConA stimulated IFN- γ production were significantly increased, whilst IL-10 neutralisation resulted in an increase in both parasite specific and ConA stimulated IFN-γ production at 12 weeks post infection, indicating direct regulatory effects. Further research into the role of IL-10 demonstrated its presence *in vitro* led to the down-regulation of mRNA transcription for IL-2 and its surface receptor, IL-4 and IFN-γ in T-cell clones isolated from experimentally infected cattle in addition to a suppression of their proliferative responses (Chitko-McKown et al., 1995).

Similar regulatory responses have been observed at the level of the local inflammatory response, with increased IL-4, IL-10 and suppressed IFN-γ mRNA transcription found within hepatic tissues taken from naturally infected cattle with chronic fasciolosis, and a direct negative correlation was observed between IL-10 and IFN-γ transcription in infected liver tissues (Mendes et al., 2013). Similarly, in experimentally infected sheep, IL-4 mRNA transcription was significantly increased and IFN-γ decreased in the hepatic lymph nodes at 23 weeks post infection (Hacariz et al., 2009). Additionally, this study observed that both IL-10 and TGF-β transcription were up-regulated in light infections, and down-regulated in heavier infections.

Transcriptomic analysis of hepatic tissue taken from single dose infected sheep at 8 weeks post infection demonstrated up-regulation of gene transcription associated with T_H2 differentiation, B-cell activation and fibrosis pathways and a down-regulation of type-1 interferon pathways (Alvarez Rojas et al., 2015).

1.3.2.3 Immune Evasion

Fasciola hepatica is capable of evading host immune responses through a variety of mechanisms.

Molecular characterisation of *F. hepatica* E/S products has demonstrated a family of cysteine proteases known as cathepsins to be the main constituents (Robinson et al., 2009). These facilitate parasite migration and nutrition through catabolism of extracellular matrix proteins and fibrinogen (Berasain et al., 1997; Dowd et al., 1995; Tort et al., 1999). The expression profile of these cathepsins alters with fluke maturity; NEJs secrete cathepsin B and cathepsin L3 predominantly, which over the course of migration and maturation are replaced by cathepsin L1 and to a lesser extent cathepsin L2, which represent the major constituents of adult *F. hepatica* E/S products (Cancela et al., 2008; Cwiklinski et al., 2015; Robinson et al., 2009). These allow the parasite to evade the host immune response, inducing apoptosis in eosinophils (Serradell et al., 2007) and macrophages (Guasconi et al., 2012), whilst E/S

antigens and specifically the cathepsins have been shown to cleave both CD4 proteins and immunoglobulins (Berasain et al., 2000; Carmona et al., 1993; Chapman and Mitchell, 1982; Prowse et al., 2002; Smith et al., 1993).

Additionally, less abundant E/S components including superoxide dismutase (SOD), glutathione peroxidase, glutathione S-transferase (GST) and peroxiredoxin (Prx) also facilitate immune evasion through oxidant scavenging activity which counteracts the oxidative killing mechanisms of ADCC (Mulcahy et al., 1999). Unsurprisingly perhaps in light of these effects, E/S products have been shown to inhibit ADCC action against NEJs by peritoneal eosinophils isolated from infected rats (Carmona et al., 1993).

In addition to E/S products, immune evasion is also facilitated by action of the tegument and its associated glycocalyx, a secreted layer of glycoproteins covering the surface of the parasite. The glycocalyx is constantly shed and replenished by dedicated cells within the tegument (Fairweather et al., 1999). This rapid turnover has been shown to inhibit ADCC by bovine eosinophils *in vitro* by reducing binding time to the parasite (Duffus and Franks, 1980; Glauert et al., 1985). Furthermore, changes are observed in the phenotype of the tegumental cells contributing to the glycocalyx as the parasite matures; type-0 tegumental cells (T0) are the major contributors to the glycocalyx of NEJs, being replaced by T1 and then T2 cells as the parasite matures (Bennett and Threadgold, 1975). This change in composition of both glycocalyx and E/S antigenic products during maturation has been shown in experimentally infected rats to facilitate further immune evasion (Tkalcevic et al., 1995; Tkalcevic et al., 1996).

1.3.2.4 Immune Modulation

In addition to aiding evasion of the host immune response, *F. hepatica* E/S and tegumental antigens (TegAg) have also been shown to effect changes in the host immune response through suppressive and modulatory effects. Parasite induced immune-modulation has been demonstrated in a wide range of important helminth species including nematode species such as the GI dwelling nematode species *Teladorsagia circumcincta* in sheep, the zoonotic cestode *Echinococcus granulosus* and the trematode *Schistosoma mansoni* in humans (McNeilly et al., 2013; Sanin and Mountford, 2015; Wang et al., 2015). In this regard *F. hepatica* is no exception. It is thought that *F. hepatica* modulates host immunity towards a non-protective response in order to extend its longevity and increase host susceptibility to re-infection (Dalton et al., 2013). These effects can be observed in chronically infected cattle where re-challenge with a trickle infection of 1300 metacercariae administered over two ten day periods (with a 13 week interval) resulted in negligible parasite specific proliferation or IFN-γ production by PBMCs (Clery et al., 1996).

In addition to the effects immune modulation has on the parasite specific immune response, a number of studies have described altered responsiveness by bovine and sheep immune cells to non-specific mitogen stimulation with ConA either in *ex vivo* cultures from either *F. hepatica* infected animals, or following treatment with *F. hepatica* antigens *in vitro* (Cervi et al., 1999; Flynn and Mulcahy, 2008a, b; McCole et al., 1998a; Zimmerman et al., 1983). Similar effects are also observed in ovine and murine immune cell responses to stimulation with LPS (Dowling et al., 2010; Hacariz et al., 2011; Martin et al., 2015; Vukman et al., 2013a) suggesting parasite induced immune modulation also causes the bystander effects on host immunity observed in *F. hepatica* infected animals (Section 1.2.2).

1.3.2.4.1 Modulatory Effects of *F. hepatica* E/S Products

In cattle, E/S antigens have been shown to induce alternative or M2 activation in blood monocyte-derived $CD14^+$ macrophages isolated from single dose infected animals when matured and stimulated *in vitro* (Flynn and Mulcahy, 2008a). As would be expected, these M2 cells exhibited increased arginase activity, reduced NO production and also produced IL-10. In mice, E/S antigens have also been shown to induce M2 activation in peritoneal macrophages taken from both infected and uninfected animals through C-type lectin receptor binding, namely the mannose receptor and Dectin-1, with increased Arginase-I (Arg1) expression and produced both IL-10 and TGF-β (Guasconi et al., 2011). Additionally, Dectin-1 mediated activation was shown to increase expression of PD-L2 cell surface receptor expression (Guasconi et al., 2015). Subsequent *in vitro* cultures of E/S activated PD-$L2^+$ M2 cells with naïve $CD4^+$ T-cells resulted in the suppression of proliferation in response to stimulation with anti-CD3 antibody, with decreased IFN-γ production and an increase in IL-10 production indicating that E/S antigen suppresses proliferative and type-1 responses indirectly through induction of M2 macrophages *in vitro* (Guasconi et al., 2015).

In addition to activation of M2 macrophages, E/S antigens have also been shown to affect the function of bone marrow derived DCs taken from uninfected mice; *in vitro* stimulation with E/S antigen resulted in an absence of cytokine production, suppression of MHC-II and CD40 surface expression and activation through TLR-binding (Falcon et al., 2010). When these DCs were co-cultured with naïve $CD4^+$ T-cells, type-2 and regulatory responses were observed characterised by IL-4, IL-5, IL-10 and TGF-β production and the increased presence of $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs.

More specifically, a number of molecules within *F. hepatica* E/S products have been shown to have immuno-modulatory properties. *In vitro* cultures of a blood monocyte derived $CD14^+$ MHC-II^+ ovine macrophage cell line (MOCL7) showed reduced NO production and an increase in both arginase and Acidic Mammalian Chitinase (AMCase) activity when cultured with either

whole fluke homogenate (LFH), E/S antigen or a purified fraction of Prx (Flynn et al., 2007a). This finding is further supported by work in mice where M2 activation was induced in response to both experimental *F. hepatica* infection and intra-peritoneal administration of E/S antigens or Prx *in vivo*, whilst *ex vivo* stimulation of macrophage cultures resulted in M2 activation with increased transcription of the AMCase enzymes yohimbine-1 (Ym1) and Fizz1 as well as arginase-I (Arg1), whilst increased IL-10 and decreased IL-12 production were also observed (Donnelly et al., 2005). Further investigation has demonstrated Prx activated M2 macrophages exhibit increased activity of Ym1, whilst co-culture with naïve $CD4^+$ T-cells resulted in increased IL-4, IL-5 and IL-13 and decreased IFN-γ production *in vitro* (Donnelly et al., 2008). Additionally, in mice cathepsin L1 has been shown to induce M2 macrophages and block LPS binding to TLR4 and TLR3, with reduced NO production and TNF-α, IL-6 and IL-12 production *in vitro* (Donnelly et al., 2010), whilst *F. hepatica* GST has been shown to suppress ConA stimulated proliferation in uninfected rat spleen cells *in vitro* and reduced NO production in peritoneal macrophages (Cervi et al., 1999). Recombinant versions of both cathepsin L1 and GST have been shown to attenuate IL-17 production and inhibit phagocytic activity by mouse DCs through TLR4 binding (Dowling et al., 2010). Additionally, fractionation of adult *F. hepatica* E/S products has identified a so-called helminth defence molecule (FhHDM-1), which once processed by cathepsin L1 produces a functional 34-residue peptide which has been shown to block the interaction of LPS and its binding receptor in mouse macrophages, resulting in a reduction of both IL-1 and TNF-α production upon intraperitoneal challenge with LPS (Robinson et al., 2011).

Recent work has also uncovered a novel low molecular weight (<10kDa) 'Kunitz type' molecule which decreased TNF-α, IL-6 and IL-12 production in bone marrow-derived murine and PBMC derived human DCs with subsequent suppression of T_H1/T_H17 differentiation and Treg induction with an associated increase in IL-10 production when co-cultured with naïve $CD4^+$ T-cells (Falcon et al., 2014). Similarly, a *F. hepatica* fatty acid binding protein (FABP) has recently been shown to inhibit LPS-TLR4 binding, TNF-α and IL-12p40 production by mouse macrophages *in vivo* following intraperitoneal administration, whilst *in vitro* stimulation of bone marrow-derived macrophages showed a suppressive effect on TNF-α, IL-1β, IL-6 and IL-12 production (Martin et al., 2015). FABP has also been shown to induce M2 activation in human macrophages (Figueroa-Santiago and Espino, 2014).

Furthermore, analysis of the recently published *F. hepatica* genome has led to the discovery of a TGF-β homologue which has been shown to be expressed in NEJs, demonstrating the potential for further as yet undiscovered immune-modulatory mechanisms (Japa et al., 2015).

1.3.2.4.2 Modulatory Effects of *F. hepatica* Surface Antigens

In addition to the modulatory effects described for E/S products, there is also evidence of *F. hepatica* inducing immune-modulation through its surface tegument.

Ex vivo culture of $CD14^+$ monocyte-derived macrophages taken from uninfected sheep and stimulated with *F. hepatica* TegAg, LFH or E/S antigen exhibited a significant increase in arginase activity when compared to LPS stimulated cells (Hacariz et al., 2011). This finding is supported by studies in mice, where intraperitoneal exposure to either TegAg or E/S antigen induced M2 activation *in vivo*, with expression of Ym1, Arg1 and IL-13 production detected and an absence of iNOS (Adams et al., 2014). *Ex vivo* culture of these cells with naïve $CD4^+$ T-cells resulted in decreased IFN-γ, IL-4 and increased IL-5 production.

In vitro stimulation of bone marrow derived mouse DCs with TegAg resulted in reduced phagocytic activity, TNF-α, IL-6, IL-10 and IL-12 production and the reduction in expression of MHC-II CD40, CD80/CD86 surface expression (Hamilton et al., 2009; Vukman et al., 2013b), whilst Vukman et al. (2013b) also observed that TegAg altered transcription of key immune regulatory molecules, including up-regulation of Suppressor of cytokine signalling (SOCS)3 and down-regulation of Mitogen-activated protein kinases (MAPKs) which are associated with suppression and stimulation of pro-inflammatory cytokine production, respectively.

Similar findings to those seen with DCs have also been demonstrated with murine MCs when stimulated *in vitro* with TegAg, subsequently failing to degranulate in response to *B. pertussis* derived LPS, which as with DCs, was associated with up-regulation of SOCS3 and down-regulation of MAPKs (Vukman et al., 2013a).

1.3.2.5 *F. hepatica* Effects on the Host Immune System and Co-infection

In addition to the investigation of parasite specific and general immune modulation, several studies have also investigated the effect of *F. hepatica* induced modulation on host immune responses to co-infecting pathogens.

Classical M1 associated macrophage responses including IFN-γ production and iNOS activity are important in the initiation of the delayed type 4 hypersensitivity response upon which both the SICCT and whole blood IFN-γ tests routinely used in the detection of *M. bovis* infection in cattle are based (Monaghan et al., 1994). Macrophages derived from $CD14^+$ blood monocytes taken from cattle experimentally infected with both *F. hepatica* and *M. bovis* displayed M2 activation with significant reduction of NO production and increased arginase activity, whilst whole blood cytokine assays stimulated with *M. bovis* derived PPD demonstrated altered IL-4 and IFN-γ production in co-infected animals over the course of the study (Flynn et al., 2007b). These findings were associated with a smaller percentage of co-infected cattle producing a positive reaction to the SICCT test when compared to those infected with *M. bovis* only.

Further investigation of the relationship between *F. hepatica* and *M. bovis* infections demonstrated a significant reduction in IFN-γ and increases in IL-4 and TGF-β production by PPD stimulated PBMC cultures isolated from co-infected animals, again compared to those infected with *M. bovis* only (Flynn et al., 2009). Claridge et al. (2012) provided both epidemiological and experimental evidence of a negative relationship between *F. hepatica* infection and *M. bovis* detection, demonstrating co-infected animals produced significantly less reaction as measured by skin fold thickness to the SICCT test than those infected with *M. bovis* only.

Further evidence of *F. hepatica* induced bystander effects in macrophages comes from experimentally infected rats, where a typical type 4 hypersensitivity reaction by peritoneal macrophages in response to Freund's complete adjuvant was found to be attenuated and phagocytic activity against the yeast *Candida tropicalis* was also reduced (Masih et al., 1996). In mice co-infection studies with *F. hepatica* and *B. pertussis*, reduction in vaccine efficacy against the latter is observed in co-infected individuals, with clearance of the bacterium from lung tissue impaired in both vaccinated and unvaccinated animals and an associated suppression of *B. pertussis* specific IFN-γ production observed spleen cells *ex vivo* (Brady et al., 1999). Further analysis of these immune-modulatory effects to *B. pertussis* vaccination were investigated through *in vivo* administration of either whole *F. hepatica* E/S antigen or purified native cathepsin L cysteine protease which resulted in suppression of *B. pertussis* specific IFN-γ production by spleen cells (O'Neill et al., 2001).

1.3.2.6 Protective Immune Responses and Vaccine Prospects

In spite of the overwhelming evidence that *F. hepatica* infection induces a non-protective response, a number of studies have demonstrated that specific components of the mammalian immune response, particularly innate cellular responses are capable of killing *F. hepatica*.

Major basic protein, the principle component of bovine eosinophilic granules, can kill NEJs *in vitro* (Duffus et al., 1980), whilst a negative correlation has shown between phytohaemagglutinin (PHA)-induced eosinophil recruitment and mean fluke length in experimentally infected animals (Bossaert et al., 2000b). Further evidence supporting the potential protective effects of cell mediated cytotoxicity comes from the demonstration of killing of hepatic stage fluke by peroxinitrite ($OONO^-$) *in vitro*, a component of the oxidative burst in M1 macrophages (Sadeghi-Hashjin and Naem, 2001).

In pigs, a species considered resistant to infection, it has been observed that fibrotic changes in the liver play a role in the attrition of migrating fluke within the parenchyma which in combination with a local infiltration of eosinophils appears to be effective in killing the parasites prior to entry into the biliary system in this species, whilst it has been suggested that

fibrotic scarring from old infections in cattle makes subsequent re-infection more difficult (Ross, 1968a). Since alternatively activated or M2 macrophages are commonly associated with fibrosis and re-modelling, and have been shown to have a close association with fibrotic changes *in vivo* it is possible these fibrotic changes constitute a potentially protective mechanism. Furthermore, it has been proposed that AMCase produced by M2 cells may also serve a protective function through degradation of parasitic chitin, although to date such action has not been demonstrated (Donnelly et al., 2008), with little evidence linking M2 macrophages to any protective effect overall. However, the reduced phagocytic and cytotoxic activities of M2 macrophages may be detrimental to the overall immune response (Golbar et al., 2013; Molina-Hernandez et al., 2015), particularly since in vaccinated cattle protective immunity is associated with a reduction in arginase activity in macrophages (Golden et al., 2010).

Further evidence of protective immunity can be observed through the adaptive immune responses of pre-sensitised hosts. Transfer of lymphoid cells from cattle and rats experimentally infected with X-irradiated metacercariae to immunologically naïve animals resulted in passive transfer of immunity and resistance to subsequent challenge in recipient animals (Corba et al., 1971). Further investigation in the murine model demonstrated protective ADCC responses could be induced by prior exposure; jejunal loops taken from *F. hepatica* infected rats displayed significant reduction in gut penetration by NEJs when challenged *ex vivo*, with maximal protective effects observed from 2-13 weeks post infection (Van Milligen et al., 1999). This was associated with increased IgE, eosinophils and MCs within the mucosal tissue itself and increased serum IgG1 antibody titre, with the latter antibody response appearing to be more important for ADCC, since NEJs recovered from the *ex vivo* model were found to be coated with IgG1 rather than IgE (Van Milligen et al., 1999), whilst immunohistology showed migrating NEJs within the intestinal walls of experimentally infected rats coated with IgG1 and IgG2a and surrounded by eosinophils (Van Milligen et al., 1998). Furthermore, Davies and Goose (1981) demonstrated rat eosinophils were capable of adhering to and killing NEJs through release of peroxidase in the peritoneal cavity of previously sensitised animals, and that this action was also associated with MC degranulation. Further experimental evidence in rats show both eosinophils and macrophages adhere to NEJs in the peritoneum in an antibody dependent manner, killing the parasite through NO production (Piedrafita et al., 2001; Sibille et al., 2004).

Whilst these findings suggest antibody response is a key component of the protective immune response in rats, in cattle and sheep the passive transfer of antiserum alone did not to confer protection against experimental infection, although in rats this was found to be the case when murine, bovine or ovine antibodies were transferred (Armour and Dargie, 1974; Corba et al.,

1971; Mitchell et al., 1981). Evidence from vaccination studies in cattle, sheep and goats suggest that where protective immune responses were induced this is associated with increased levels of high avidity IgG2 antibody titres, perhaps suggesting this isotype is more important in ADCC mechanisms against *F. hepatica* in ruminants compared to murine model hosts (Maggioli et al., 2011; Mulcahy et al., 1998; Villa-Mancera et al., 2014).

A number of vaccine trials have been attempted over the years in sheep and cattle. Early work focussed on the administration of γ-irradiated metacercariae to give naïve animals immunity through an attenuated primary infectious challenge following on from work performed by Jarrett et al. (1960) which established an effective commercial vaccine against the bovine nematode *Dictyocaulus viviparus* using similar techniques. Whilst a protective effect was demonstrated following administration to cattle (Nansen, 1975), no such effect was observed in sheep (Campbell et al., 1978; Creaney et al., 1995).

Subsequent efforts have focussed on the induction of protective immune responses through stimulation by specific *F. hepatica* antigens through parenteral delivery. In light of the immune responses observed in sheep and cattle, induction and stimulation of a cell mediated immune responses appear to be an important feature of protective immunity, whilst the inhibition of parasite-induced immune-modulation is a key target also. As a consequence, many vaccination studies in cattle, sheep and goats have focussed on attempting to induce a type-1 immune response against antigens deemed vital for fluke survival, including those immuno-modulators previously identified such as cathepsin L proteases, GST, Prx in addition to a number of other molecules such as *F. hepatica* haemoglobin delivered with one of a number of adjuvants designed to induce either T_H1 stimulation such as Freund's complete/incomplete adjuvant (FCA/FIA) and Quil-A, or a balanced T_H1/T_H2 response such as Montanide™ (Golden et al., 2010; Mulcahy et al., 1998; Toet et al., 2014; Villa-Mancera et al., 2014). These studies have demonstrated mixed efficacy, with wide ranging levels of protection from zero up to 72% protection in cattle and up to 86% in sheep (Toet et al., 2014).

In addition to inducing a protective immune response there are a number of additional challenges to be overcome in the development of a commercial vaccine which include ease and cost of manufacture. Native antigens have been used in the manufacture of a commercial vaccine against the parasitic nematode *Haemonchus contortus* (Bassetto et al., 2014). However, this was performed more out of necessity rather than for convenience as the reliance upon anthelmintics to control *H. contortus* had become untenable as a result of multi-drug resistance. It is unlikely therefore development of vaccines based on native antigens would be pursued for *F. hepatica* unless the situation regarding anthelmintic control becomes equally dire. Recombinant forms of several antigen candidates have been successfully manufactured and trialled in vaccine studies with varied levels of success. A recombinant form

of cathepsin L1 produced using the commonly used yeast *Pichia pastoris* has been shown to have cross reactivity with immunoglobulins generated against native antigen (Kuerpick et al., 2013), whilst vaccination studies using recombinant forms of the cathepsin L1 molecule have shown significant levels of protection against infection in cattle, sheep and goats (Golden et al., 2010; Villa-Mancera and Mendez-Mendoza, 2012; Villa-Mancera et al., 2014). In cattle, following vaccination with a recombinant version of the cathepsin L1 protease and subsequent exposure to fluke contaminated pasture under natural conditions a 48% reduction in fluke burden was observed relative to un-vaccinated control animals (Golden et al., 2010). Whilst such findings show the potential for vaccines in helping to control *F. herpatica*, it is also clear further investigation and development is required before these become a commercially viable commodity.

1.4 Thesis Aims

Although a great deal of work has been performed looking into the immune responses of experimentally infected cattle, little is known of the immune responses of cattle when exposed to natural infection, with nothing known of the early immune responses in this setting. It is important to understand such immune responses from the context of not only vaccine development, but also from the perspective of the general immune response and the potential bystander effects resulting from *F. hepatica* induced immune modulation. This thesis sets out to investigate the immune responses of cattle naturally exposed to *F. hepatica* both in primary infection (Chapter 4) and chronic infection (Chapter 5). In addition, we present data on the validation of a composite faecal egg count method which was devised principally to provide a screening tool to identify potential study farms for subsequent studies, but which also has potential value in the diagnosis and management of fasciolosis in groups of cattle, particularly non-lactating animals.

Chapter 2

Materials and methods

This chapter contains experimental protocols that were used throughout the thesis. Specific details are included in each chapter. Where relevant, data have been included in this chapter, if they were involved in the decision making process.

2.1 Ethical Approval and Data Protection

Prior to commencement of the study, all planned experimental work involving animals was submitted to, and approved by, the University of Liverpool ethics committee. All participants remained anonymous for the duration of the study. Personal details were removed from datasets and stored separately in an encrypted file on a secured network, in keeping with the Data Protection Act 1998. Samples from cattle were collected under home office license (HOL PPL40/3621).

2.2 Sample Size Calculation

As part of the ethical review process a sample size calculation was performed. Previously acquired data from a study observing immune responses in cattle to infection with the protozoan parasite *Neospora caninum* were used to determine the minimum sample size required to detect a significant difference in cytokine production pre- and post-infection, specifically interferon(IFN)-γ. This data was generated using protocols outlined in Rosbottom et al. (2007). Briefly, 15 Holstein-Friesian calves were inoculated via intravenous injection with 10^7 *N. caninum* ("Nc Liverpool") tachyzoites. Peripheral blood mononuclear cells (PBMCs) were harvested at week 0, 1, 2, 3, 4, 6 and 8 of infection. Concanavalin A (ConA) stimulated IFN-γ production was quantified in *ex vivo* culture supernatants using a commercially available ELISA (figure 2.1). Since we intended to use the same laboratory protocols in our study, this gave an approximation of the order of magnitude of change in cytokine production we might expect to see in response to infectious challenge.

Measurements for time point 0 were taken as pre-infection values, whilst all other measurements were compiled and categorised as post-infection. This approach was adopted since the variation in combined post infection IFN-γ responses from all time points was anticipated to better reflect those of a natural infection.

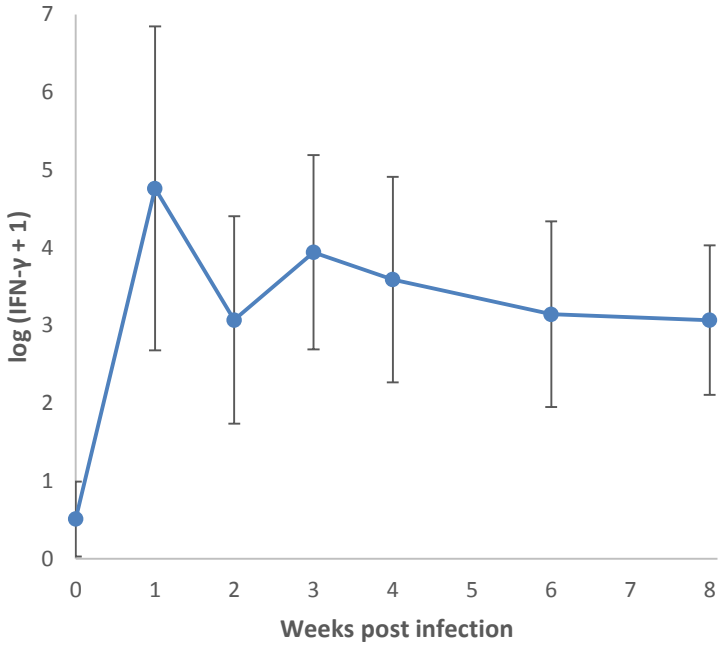

Figure 2.1: Mean log(+1) IFN-γ responses of Bovine PBMCs *in vitro* to ConA following experimental infection with *N. caninum* (n=16) (±SD).

Following log+1 transformation to normalise distribution, pre-infection (x_0) values were subtracted from corresponding post-infection values (x_i) for each animal at each time point to give the difference (x_{bar}):

$$x_{bar} = \log(x_i + 1) - \log(x_0 + 1)$$

Mean and standard deviations were calculated from these values (n=84) and used to determine a minimum sample size, which was determined to be the point at which the lower critical limit of x_{bar} exceeded the upper critical limit of x_0 (figure 2.2). From this, we determined that a sample size of between 12-30 animals should be sufficient to detect a significant increase in cytokine responses in PBMC cultures *in vitro* from animals pre- vs. post-exposure.

2.3 Farm and Animal Recruitment

To recruit farms, bulk milk tank (BMT) *F. hepatica* antibody ELISAs (Section 2.6) were offered to local dairy farms in Cheshire and North Wales free of charge. All farms who submitted bulk milk tank samples were given results, with a positive cut-off PP-value of ≥27, and further categorisation of positive results into low, medium and high values, defined as PP-values from ≥27-50, ≥50-99 and ≥100, respectively (McCann et al., 2010; Salimi-Bejestani et al., 2005a).Farms were recruited, based on a positive bulk milk tank result and willingness to participate in the study. The farms are henceforth referred to as “A”, “B” and “C” (Chapter 4) and “D” (Chapter 5). All were commercial dairy farms, with comparable herd sizes, yield, fertility etc. to the national average of 126 dairy cows (Baker, 2015), and at the time of the

study were not experiencing any major herd health issues. The initial bulk milk tank results for each farm are given in table 2.1.

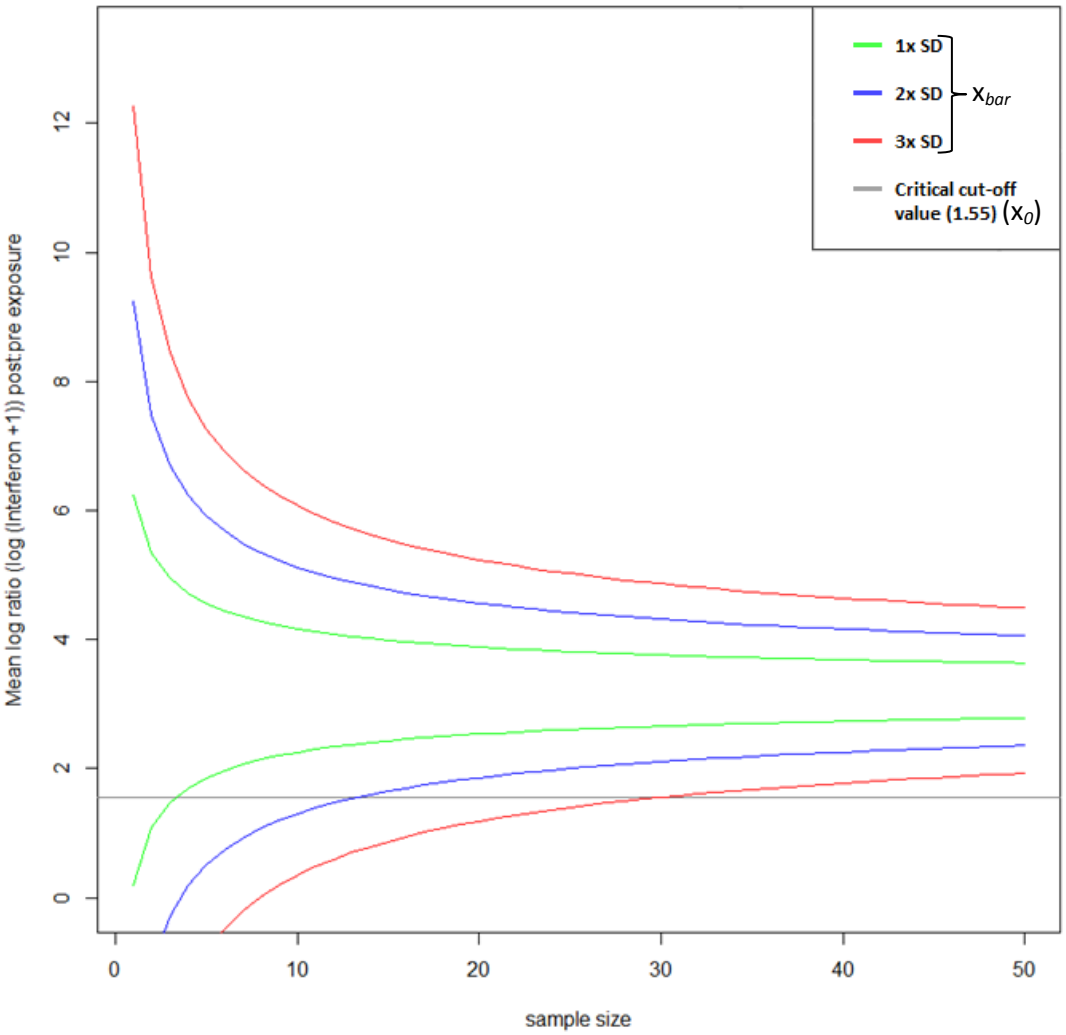

Figure 2.2: Calculated critical limits for differences in log(+1) transformed data pre:post exposure (critical limits calculated as multiples of standard deviation [SD]). This figure shows the relationship between sample size and standard deviation of x_{bar}. Standard deviation (SD) decreases with increasing sample size. Using critical limits defined as multiples of the sample standard deviation (1x, 2x and 3x SD) and comparing these to the upper critical limit of x_0 (1.55), the lower critical limit of x_{bar} exceeded this value at a sample size ≥12 animals for 2x SD and ≥30 animals at 3x SD.

2.3.1 Primary Exposure Study (Chapter 4)

To assess the changes in immune responses over the course of a primary infection with *F. hepatica*, we recruited groups of replacement dairy heifers from 3 local dairy farms. Animals were aged 90-377 days old (mean=218.5, SD ±62.0), and had not been previously turned out to grass. They were therefore considered to be immunologically naïve, having had no previous exposure to *F. hepatica*, confirmed by negative serum antibody ELISA and fluke egg counts prior to turn out (Sections 2.6 & 2.7.2.2). A total of 44 animals were recruited, with 17, 17 and 8 animals from farms A, B and C respectively. Animals were sampled prior to turn-out, then on a monthly basis over the course of the grazing season of 2013 and when they were housed in the autumn. On farms A and C, it was not possible to sample all animals at each time point due

to movement between management groups. At the initial sampling an additional heparinised blood sample taken and submitted to the AHVLA to be assessed for BVD using the E^{rns}-antigen ELISA to check for persistently infected animals since this may have impacted on immune responses to other infectious agents subsequent *in vitro* assays (Radostits and Littlejohns, 1988).

Monthly feedback was given in the form of calf weight measurements and BMT results. Fluke infection status in the calves was not reported, as this may have resulted in treatment by the farmer and consequently affected the results of the study; withholding of this information was approved under the ethical review process and agreed with farmers upon enrolment. Final antibody titres and infection status of each animal was given at the completion of the study so that farmers could give targeted treatment to infected animals.

Table 2.1: Bulk milk tank F. hepatica ELISA results for recruited farms.

Farm ID	BMT ELISA result	Date
A	43.1	23/11/2012
B	53.8	04/12/2012
C	114	22/11/2012
D	73.9	05/02/2013

2.3.2 Chronic Infection Study (Chapter 5)

To assess the immune responses to naturally acquired infection in adult cattle, monthly visits were made from May to October 2014 to a commercial dairy herd (Farm "D"). This farm was chosen since the BMT ELISA result was high (table 2.1) indicating a high proportion of infected animals in the herd (Salimi-Bejestani et al., 2005a). At each visit lactating dairy cattle due to be dried off in the following month were sampled. Infection status was fed back to the farmer after each visit, with animals testing positive on fluke antibody ELISA (Section 2.6) treated at drying off with TCBZ ahead of calving. All animals were re-sampled following calving, including previously sero-negative animals, which served as a control group to account for a potential impact of pregnancy on immune responses (Mallard et al., 1998). A total of 27 animals were recruited for this study, with 17 animals re-sampled following calving, of which 10 had received treatment with TCBZ to clear infection with *F. hepatica*. All animals were kept under their normal farm management conditions throughout the study, with both lactating and dry cows remaining at pasture throughout the grazing season.

2.4 Sample Collection

Animals were gathered and handled using the normal on farm handling systems operated by farm personnel to reduce stress and risk of injury to the cattle. Halters were used for additional restraint where necessary.

2.4.1 Blood Sampling

Blood samples were collected via jugular venepuncture; total blood volume taken never exceeded 40ml. Whole blood was collected into two EDTA coated 10ml vaccutainers. Serum samples were collected in plain 10ml vaccutainers and transported back to the laboratory at room temperature.

Once clotted, serum tubes were centrifuged at 2,000g for 5 minutes to separate out the serum fraction, which was aliquoted into eppendorfs and refrigerated at 4^{o}C ahead of serum antibody ELISA (Section 2.6), and then frozen at -20^{o}C for long term storage.

2.4.2 Faecal Sampling

Faecal samples were collected rectally from each individual animal at each sampling. Samples were transferred to pots and transported to the laboratory at room temperature where they were refrigerated at 4^{o}C ahead of further processing (Section 2.7).

2.4.3 Bulk Milk Tank (BMT) Sampling

Bulk milk tank samples were collected and put into pots containing bronopol preservative tablets (Advanced Instruments Inc., Norwood USA).The tank was agitated to ensure milk was homogenised ahead of sample collection. A jug was used to fill the sample pot from the tank rather than using the sample pot directly to avoid contaminating the bulk tank with preservative. The sample pot inverted a number of times to ensure the preservative tablet was dissolved ahead of transportation back to the laboratory at room temperature. Samples were centrifuged at 2,000g for 5 minutes to separate out the lipid and non-lipid fractions, with only the non-lipid fraction used in the ELISA to improve the consistency of the assay (Salimi-Bejestani et al., 2005a). Samples were aliquoted into eppendorfs and refrigerated at 4^{o}C ahead of antibody ELISA (Section 2.6), and then frozen at -20^{o}C for long term storage.

2.4.4 Weight Tape

In the primary infection study (Chapter 4), weight measurements were taken to assess productivity and performance using a Holstein weight tape (Nasco, Fort Atkinson USA) to estimate weight in kilograms from heart girth measurement, which has been shown to have a linear relationship with weight in growing cattle (Heinrichs, 1914). Whilst this data was recorded, ultimately no significant association was found with either infection or immunological responses (*APPENDIX A*)

2.5 Preparation of *F. hepatica* Antigens

Three different *F. hepatica* antigens were prepared: Excretory/Secretory (E/S) antigen, Tegumental antigen (TegAg) and Somatic antigen (SomAg). All antigens were prepared sequentially under aseptic conditions using sterile reagents. E/S-antigen was produced using live fluke, following which TegAg was prepared, then finally SomAg.

2.5.1 Excretory-Secretory (E/S) Antigen

Fluke-infected livers were collected fresh from the abattoir and transported back to the laboratory at room temperature. Adult fluke were harvested by incising the bile ducts and removing live adult fluke using atraumatic tissue forceps. Fluke were placed individually in 1ml RPMI (Sigma-Aldrich, St. Louis USA) with 25µg/ml genticin (Invitrogen Life Technologies, Grand Island USA) in 24 well tissue culture plates and incubated at 37^{o}C for 2 hours to purge caecal contents. At completion of incubation, fluke were assessed for viability, with dead or damaged specimens discarded. The remainder were then washed three times in copious volumes of warm (37^{o}C) D-PBS (Sigma-Aldrich, St. Louis USA). Live fluke were then placed in 25cm^{2} tissue culture flasks (VWR, Radnor USA) in RPMI with 25µg/ml genticin, 0.5ml per fluke, 10 fluke per flask, and incubated overnight at 37^{o}C in 5% CO_2. Media containing E/S products were collected the following day and centrifuged at 600g for 20 minutes at 4^{o}C to separate out eggs and other solid debris then aliquoted into eppendorfs for storage at -80^{o}C.

2.5.2 Tegumental Antigen

Following E/S antigen preparation, fluke were removed from tissue culture flasks and washed three times in copious volumes of D-PBS and placed in a plug sealed 50ml tissue culture flask (Thermo Fisher Scientific, Waltham USA) in D-PBS with 1% Nonidet P-40 (BDH Chemicals, Poole UK) at a volume of 1ml/fluke. The flask was placed on ice and rocked gently for 1 hour, after which the supernatant containing tegument antigen was harvested. For samples intended for immediate use, Pierce® Detergent Removal spin columns (Thermo Fisher Scientific, Waltham USA) were used according to the manufacturer's specifications to remove the NP-40 detergent from the solution; in a sterile hood, columns were un-capped and placed in 15ml falcon tubes before being centrifuged at 1000g for 2 minutes to remove storage solution. Four millilitres of wash/equilibration buffer was then added to each column and centrifuged at 1000g for 2 minutes. This wash step was repeated 3 times. Following this, 1000µl of TegAg containing 1% NP-40 solution was pipetted slowly onto the resin bed within the column and incubated for 2 minutes at room temperature then centrifuged in fresh falcon tubes at 1000g for 2 minutes to collect the NP-40 free TegAg. These samples were aliquoted for storage at -80^{o}C ahead of use.

Remaining TegAg containing 1% NP-40 was also aliquoted and stored at -80^{o}C. Technical advice from Thermo Fisher scientific suggested that freezing samples prior to detergent removal would not adversely affect the procedure.

2.5.3 Somatic Antigen

Tegument depleted fluke (Section 2.5.2) were washed three times in copious volumes of D-PBS and snap frozen overnight at -80^{o}C. The following day, fluke were removed from the freezer and homogenised in a small volume of D-PBS with a sterile pestle and mortar. The fluke homogenate was transferred to a 50ml falcon tube and diluted to a concentration of 0.5ml/fluke in D-PBS and left to stand overnight at 4^{o}C. The resulting supernatant was pipetted off and centrifuged at 12,000g for 30 min at 4^{o}C to further remove dense particulate. Resulting supernatant was then filter sterilised with a 0.22µm pore filter (Merck KGaA, Darmstadt Germany) before aliquoting and storage at -80^{o}C ahead of use.

2.5.4 Coomassie Plus (Bradford) Assay

Protein concentrations were calculated using the Coomassie Plus (Bradford) assay following a standardised microplate protocol as per manufacturer's recommendations (Thermo-scientific technical manual 0229.6, Microplate Procedure B). Bovine serum albumin (BSA) stock standard (Thermo Fisher Scientific, Waltham USA) was diluted to give a standard curve of protein concentrations ranging from 2.5-25µg/ml by diluting BSA stock in distilled water as per manufacturer's recommendations (*APPENDIX B.1*). Samples were serially diluted in distilled water to 1:100, 1:200 or 1:400. Standards and samples were pipetted in duplicate into a flat clear bottomed 96-well plate, 150µl per well. Bradford reagent (Sigma-Aldrich, St. Louis USA) was added, 150µl per well, to each standard and sample dilution using a multichannel pipette taking care to ensure thorough mixing. The plate was incubated for 5-10 minutes at room temperature before absorbance was measured at 595nm on a Tecan infinite F50 plate reader. Sample concentrations were determined from linear regression analysis applied to BSA standards using untransformed OD values. In cases where sample OD values exceeded the maximum 25 µg/ml standard the result was discarded. Protein concentrations for TegAg and SomAg are given in table 2.2.

2.5.5 Endotoxin Detection Assay

SomAg and TegAg samples intended for use in tissue culture were checked for endotoxin using a commercially available kit (Thermo Fisher Scientific, Waltham USA) following manufacturer's specifications (Thermo-scientific technical manual 2445.1). Briefly, standards and samples were incubated in duplicate with Limulus Amebocyte Lysate (LAL) and a chromogenic

substrate to facilitate colour development. Standard and sample absorbance was measured at 405nm and concentration determined through linear regression on untransformed standard OD values. Concentrations of endotoxin in SomAg and TegAg were found to be 0.24 and 0.0026 EU/ml respectively at antigen concentrations intended for use in tissue cultures. These values were below previously determined threshold limits of LPS contamination (Ryan, 2008).

2.6 *F. hepatica* Antibody ELISA

Fluke antibody ELISAs were performed following previously validated protocols for serum and BMT samples (Salimi-Bejestani et al., 2005a).

2.6.1 Plate Coating

Immulon 2HB flat bottomed 96-well plates (Thermo Fisher Scientific, Waltham USA) were coated with E/S antigen at a concentration of 0.5µg/ml diluted in 0.1M carbonate coating buffer (pH 9.6) (*APPENDIX B.4*), 100µl per well. Plates were covered with adhesive plate sealers (STARLAB, Hamburg Germany) and incubated overnight at 4^{o}C.

2.6.2 Wash Step

The following day, plates were emptied of coating solution and washed with a PBS 0.05% tween-20 wash buffer (*APPENDIX B.5*). This involved 3 sequential washes, with plates left filled with wash buffer for 5 minutes at the end of the third wash. This wash cycle was then repeated after which wash buffer was then removed from the plates by flicking the contents out briskly and tapping the plate onto absorbent material to remove residual material and ensure no bubbles remained in the test wells.

2.6.3 Blocking Step

Following washing, 200µl of 2% skimmed milk powder blocking buffer (*APPENDIX B.5*) was added to each well. Plates were covered with adhesive plate sealers and incubated at 37^{o}C for 1 hour, following which plates were washed as previously described (Section 2.6.2).

2.6.4 Sample Preparation and Incubation

Serum controls (high-, mid-positive and negative) were diluted 1:800 in blocking buffer and added in quadruplicate to the ELISA plate, 100µl per well, with four wells loaded with blocking buffer alone to serve as blanks, of which two were designated as conjugate blanks for use in the calculation of PP-values (Section 2.6.5).

Serum samples were used at a dilution of 1:800 (Salimi-Bejestani et al., 2005b). BMT samples were added directly to the ELISA plate without dilution (Salimi-Bejestani et al., 2005a). Plates

were covered and incubated at 37°C for 1 hour. Following incubation, plates were washed as previously described (Section 2.6.2).

2.6.5 Antibody Detection

Fasciola hepatica antibody detection was facilitated by HRP-conjugated anti-bovine IgG mAb (clone IL-A2) at a dilution of 1:100,000 in blocking buffer (Salimi-Bejestani et al., 2005b), 100µl per well, with the exception of the designated conjugate blank wells, which were instead loaded with 100µl of blocking buffer. Plates were covered and incubated at 37°C for 1 hour following which plates were washed as previously described (Section 2.6.2).

For colour development, TMB (Interchim, Montluçon France) was used as substrate, 100µl per well, following which plates were incubated for 20 minutes in the dark. The reaction was stopped by adding 0.5M HCl, 100µl per well. Absorbance was measured at 450nm and sample Percent Positivity (PP) was calculated as follows:

$$Percent\ positive\ (PP) = \frac{(Mean\ OD\ of\ test\ sample - Mean\ OD\ of\ conjugate\ blank)}{(Mean\ OD\ of strong\ positive\ control - Mean\ OD\ of\ conjugate\ blank)} \times 100$$

Cut-off PP values were chosen depending upon the requirements of the test; for the primary exposure study, a positive cut-off value of 20 was used to give a test sensitivity of 95% and specificity of 99%. For BMT samples, a cut-off value of 27 was used giving a sensitivity of 96% and specificity of 80% (Salimi-Bejestani et al., 2005a).

2.7 Faecal Egg Counts

Faecal samples were assessed for both nematode and trematode eggs using standard laboratory techniques.

2.7.1 McMaster Method for Detection of Nematode Eggs

Three grams of faeces were weighed out into a sample pot to which 42ml of saturated salt (NaCl) solution was added. Glass beads were added and the sample shaken to homogenise the sample. This was passed through a sieve, with the filtrate collected in a fresh container. This was loaded into each chamber of the McMaster slide. It was necessary to continually agitate the sample while performing this step to ensure an even distribution of eggs throughout the solution.

The McMaster slide was microscopically examined at 10-20x magnification and cumulative egg counts recorded from both chambers, counting only eggs within the etched area. This was multiplied by 50 to give the number of eggs per gram of faeces. *Nematodirus* spp. eggs were

distinguished by their size and morphology, from other species and counted and recorded separately.

2.7.2 Sedimentation Methods for Detection of *F. hepatica* Eggs

Two separate methods were employed to evaluate fluke egg counts during this project; a standard sedimentation method (Chapters 3 & 4)(ANON, 2007), and a commercially available kit (Chapter 5) (Flukefinder®).

2.7.2.1 Individual Samples

Ten grams of faeces were weighed into a sample pot and mixed with water to homogenise. The mixture was then passed through three stacked sieves of aperture diameters 750µm, 150µm and 38µm. These were stacked with the largest aperture (750µm) at the top and the smallest (38µm) at the bottom. Sieves were washed through with copious amounts of water and any residual clumps of faecal material were broken up. Once the water exiting the bottom sieve ran clear, the top sieve was removed and the process repeated with the remaining two sieves. Following this, the material collected in the 38µm aperture sieve was transferred to a 500ml glass beaker by rinsing with water to ensure that all sediment was transferred. All three sieves were washed thoroughly ahead of use with other samples to ensure no cross sample contamination occurred.

The sample was diluted to 500ml with water and left to stand for 4 minutes. The resulting supernatant was then decanted to leave the sediment at the bottom undisturbed. Another 500ml of water was added and left for a further 4 minutes. This sedimentation process was repeated until the supernatant was clear, at which point it was poured off for the last time to leave a minimum residual volume, which was either transferred directly to a petri dish for counting or alternatively to a falcon tube for storage at 4^{o}C. To aid counting, a few drops of methylene blue were added to the sample in the petri dish to help distinguish the fluke eggs from background material. Samples were examined under a dissection microscope at 4-10x magnification and total number of eggs counted.

2.7.2.2 Composite Samples

Composite sample analysis followed the same procedure as that detailed for individual samples (Section 2.7.2.1) with the following modifications:

Composite samples comprised of 5 grams from ten individual samples to give a total of 50 grams of faeces. It was necessary when processing these samples not to overload sieves by adding too much material at once. The sedimentation process was the same as that detailed for individual samples (Section 2.7.2.1), although typically more washes were required. When

counting, in some instances it was necessary to split the contents over 2-3 petri-dishes to ensure accurate counts could be made.

2.7.2.3 Fluke Egg Detection using Flukefinder®

Flukefinder® (Soda Springs USA) is a commercially available kit which uses a modified sedimentation method and follows the same principle as that in Section 2.7.2.1. Tests were performed according to manufacturer's recommendations; three grams of faeces were weighed out into a fresh sample pot and mixed with tap water to form homogenous slurry. The mixture was added to the assembled fluke finder and liquid passed through. The retentate was further washed with additional water, taking care not to over fill the units. This was repeated until the fluid exiting the Flukefinder® ran clear. The retentate in the bottom sieve was washed into a fresh beaker and transferred to a 15ml Falcon tube for sedimentation. The Flukefinder® units were thoroughly cleaned after each use to avoid cross contamination. The falcon tube was filled to 15ml total with water and the contents left to stand for 3 minutes. After this time the supernatant was discarded, whilst taking care not to disturb the sediment at the bottom of the tube. This process was repeated until the supernatant was clear, after which the supernatant was discarded and the sediment transferred to a petri dish for counting under a dissection microscope. Methylene blue was added as a background stain to help identify fluke eggs.

2.8 Peripheral Blood Mononuclear Cell (PBMC) Purification

Peripheral blood mononuclear cell (PBMC) purification was performed in a lateral flow hood under sterile conditions. EDTA treated whole blood samples were transferred into 50ml falcon tubes, with a small amount retained for total leukocyte enumeration and peripheral blood smears (Section 2.11.1). The remainder was used for PBMC isolation using Optiprep™ (Sigma-Aldrich, St. Louis USA).

Optiprep™, stored at 4°C, was inverted a number of times following removal from the refrigerator to ensure the solution was mixed ahead of use, following which 1.3ml per 10ml of blood was added and mixed thoroughly with the blood sample and 1ml of 20mM tricine-buffered saline (*APPENDIX B.6*) layered over the top of the blood/optiprep mixture to create a phase fluid interface from which PBMCS could be harvested. Samples were then centrifuged at 1000g for 35min at 20°C, after which the centrifuge rotor was allowed to stop without braking. Samples were then transferred carefully back to the hood. Using a sterile Pasteur pipette the middle fluid layer containing PBMCs and serum was harvested down to approximately 0.5ml above the cell pellet. The harvested medium containing PBMCs was transferred to a fresh 50ml falcon tube and diluted with a minimum of two volumes of PBS with 0.1% EDTA wash buffer

(Lonza, Bazel Switzerland) up to a maximum total volume of 20ml and centrifuged at 350g for 8min at 20^{o}C with the brake off to pellet PBMCs.

A haemolysis step was performed to remove any contaminating erythrocytes; supernatant was poured off and cells re-suspended by gentle tapping. Two millilitres of 0.9% NH_4Cl erythrocyte lysis buffer was added to samples and gently agitated at room temperature for 1 minute after which 20ml of PBS EDTA wash buffer was added and samples centrifuged with the brake off at 150g for 8min at 20^{o}C. Supernatant was again discarded and pelleted PBMCs re-suspended in the residual fluid. This wash and spin process was repeated twice after which the re-suspended PBMC pellet was diluted in 5ml cell culture medium consisting of RPMI supplemented with 10% heat inactivated foetal calf serum (Sigma-Aldrich, St. Louis USA) and penicillin and streptomycin (Sigma-Aldrich, St. Louis USA) at a concentration of 100µg/ml.

Cell counts were determined via haemacytometer using trypan blue exclusion to assess cell viability at a 1:50 sample dilution in PBS EDTA. In the majority of cases, the percentage of dead cells was very low (less than 10%). PBMC counts/ml were then calculated as follows:

$$Cells\ per\ ml = \frac{total\ count}{number\ of\ complete\ 4x4\ grids\ counted} \times 10^4 \times sample\ dilution$$

Based on these counts, culture medium was added to adjust cell concentration to 2 x 10^6 PBMCs/ml for cell culture.

2.8.1 PBMC Proliferation Assays

Following adjustment of PBMC concentration to adjusted to 2x10^6 per ml in culture medium (RPMI with 10% FCS and penicillin and streptomycin at 100µg/ml), samples were pipetted out into U-bottomed 96-well tissue culture plates (Corning Life Sciences, Corning USA) , 100µl per well, to which a further 100µl of either culture medium, mitogen Concanavalin A (ConA)(Sigma-Aldrich, St. Louis USA), *F. hepatica* TegAg (Section 2.5.2) or SomAg (Section 2.5.3) were added at specified concentrations (table 2.2) to give a total volume of 200µl of culture medium and 2x10^5 PBMCs per well. Antigen concentrations were chosen to match previous investigations (Bossaert et al., 2000b).

Stock ConA solutions were prepared ahead of use by dissolving freeze dried mitogen in culture medium to a concentration of 1000µg/ml before aliquoting and storage at -20^{o}C. *F. hepatica* antigen stocks were aliquoted and stored at -80^{o}C ahead of use. Each sample was set up in culture with ConA, SomAg, and TegAg in triplicate. Outer wells on each plate were left empty to reduce evaporation of culture medium on incubation.

Table 2.2: Mitogen and antigen culture conditions/dilutions.

	Concentrations (µg/ml)		Dilution (1:x)
	Stock	Culture	RPMI w/10% FCS
Mitogen			
ConA	1000	5	99
***F. hepatica* antigen**			
SomAg	5677	25	112.5
TegAg	1577	25	29.2

Lids were placed on the cell culture plates and these were incubated for 5 days at 37°C in 5% CO_2. Plates were checked daily under the microscope for signs of cell viability and proliferation and to check for bacterial and/or fungal contamination. In some instances, PBMC cultures contained *Trypanosoma theileri*, a common non-pathogenic parasite of cattle. Wells containing these parasites were noted and the data excluded from further calculations in the acute infection study (Chapter 4). In the chronic infection study (Chapter 5) an additional 20µl of serum from an animal previously identified as having trypanosomes was added to the culture wells to control their propagation through complement fixation. This reduced the number of trypanosomes in most instances, although contaminated wells were still noted and excluded from further analysis.

After 5 days, the PBMCs were pulsed with 1µCi of [H^3] tritiated thymidine (Perkin Elmer, Boston USA), 20µl per well, diluted in culture medium. PBMCs were then incubated for a further 5 hours at 37°C at 5% CO_2. Work with [H^3] tritiated thymidine was carried out under standard radiation working safety conditions.

At the end of incubation, plates were loaded onto a Tomtec 96 Mach III cell harvester and flushed through with distilled water with contents collected onto glass filter mats (Perkin Elmer, Boston USA). The harvester was put through a wash/harvest cycle using a blank plate between samples to ensure no residual material was carried over from one plate to the next. Following this filter mats were air-dried, embedded in scintillation wax (Perkin Elmer, Boston USA) and enclosed within cellophane sample bags (Perkin Elmer, Boston USA). Filter mats were loaded into cassettes and read on a beta-counter (Perkin Elmer, Boston USA) using a standard count protocol for detection of β-particle emission, with scintillation counts recorded for each well for 15 seconds.

Counts were normalised by dividing mean antigen/mitogen β-particle decay counts by the corresponding mean medium control count to give the "Stimulation index" (SI) as a relative measure of PBMC proliferation.

2.8.2 PBMC Cytokine Assays

For cytokine cultures, PBMCs at a concentration of $2x10^6$/ml in culture medium were added to flat bottomed 24 well cell culture plates (VWR, Radnor USA) at a volume, 1ml per well. Three

separate cultures per sample were prepared, to which 1ml of either culture medium, the mitogen ConA or *F. hepatica* SomAg was added at the same concentrations used for the proliferation assays (table 2.2). Plates were incubated at 37^{o}C, 5% CO_2 for 48 hours. Samples were checked at 24 hours for evidence of bacterial and/or fungal contamination. No trypanosomes were detected at 48 hours.

Following the 48 hour incubation period, supernatants were collected; each sample was pipetted up and down in the well a number of times to re-suspend non-adherent PBMCs. Samples were aliquoted into 1.5ml eppendorfs and centrifuged at 800g for 10min after which supernatants were removed to a fresh eppendorf and stored at -20^{o}C for cytokine analysis (Section 2.9). Two hundred microliters of RNAlater (Sigma-Aldrich, St. Louis USA) was added to the remaining cell pellets and refrigerated overnight at 4^{o}C as per manufacturer's recommendations before long-term storage at -20^{o}C ahead of RNA extraction and qPCR (Section 2.10).

2.9 Cytokine ELISAs

Cytokine ELISAs were performed on culture supernatants (Section 2.8.2). To try and maintain consistency, all samples from one individual were tested on the same plate. Care was taken to avoid repeated freeze thawing of samples which could affect results. Samples were thawed at 4^{o}C overnight ahead of the ELISA being performed.

2.9.1 Interferon-γ

Interferon-γ was measured in supernatants using a Bovine IFN-γ specific ELISA Kit (AbD Serotec, Raleigh USA) following manufacturer's protocol (QIFUMCA5638KZZ Bovine IFN-g ELISA Kit Insert IFU, AbD Serotec). Flat bottomed 96 well ELISA plates were coated with 100µl per well of anti-bovine IFN-γ monoclonal antibody (mAb) diluted 1:200 in coating buffer and incubated at 4^{o}C overnight. The following day plates were washed three times in wash buffer consisting of 0.2M NaCl with 0.05% Tween-20. Blocking buffer consisting of 4% BSA (Sigma-Aldrich, St. Louis USA) in PBS, 200µl per well. The plate was covered with a plate sealer and incubated at room temperature for 1 hour. During the incubation for the blocking step, samples and standards were prepared. Freeze dried Interferon-γ standard was initially re-constituted in 650µl of distilled water to form a stock solution from which standard concentrations were prepared by diluting in wash buffer (*APPENDIX B.2*) before being loaded onto a 96 well mixing plate. Samples were prepared by pipetting out onto a mixing well. Due to the wide range of sensitivity of this ELISA kit, no dilution of samples was necessary.

Following incubation with blocking buffer, wells were emptied of contents and washed as described previously. Standards and samples were then added in duplicate, 100µl per well.

Plates were covered with a plate sealer and incubated at room temperature for 1 hour. Following this incubation, plates were emptied of contents and washed as described previously, after which detection antibody diluted to 1:500 by adding 20µl to 10ml wash buffer was added 100µl per well, plates covered with adhesive plate sealer and incubated for 1 hour at room temperature, then washed as previously described. Streptavidin:HRP was added having been diluted to 1:1000 from stock in wash buffer at 100µl per well and incubated for 1 hour at room temperature, then washed again as previously described. TMB was added at a volume of 100µl per well and plates were incubated for 15 minutes in the dark to allow colour development after which the reaction was stopped with 0.5M HCl at a volume of 100µl per well and plates read on plate reader at 450nm. To calculate sample concentration, the standard curve was constructed using log transformed OD values and concentration values and point to point regression performed (*APPENDIX B.2*).

2.9.2 Interleukin-4

Two different ELISAs were used for the quantification of IL-4 for the two different studies; a commercially available bovine Interleukin-4 ELISA kit (AbD Serotec, Raleigh USA) was used to evaluate samples from the primary infection study (Chapter 4), whilst a standardised ELISA protocol (Hope et al., 2005) was used to assess samples from the chronic infection study (Chapter 5) since the Serotec kit was not available for purchase at the time.

2.9.2.1 IL-4 Serotec ELISA kit

Interleukin-4 was measured in thawed culture supernatants (Section 2.8.2) using the commercially available Bovine Interleukin-4 Specific ELISA Kit (AbD Serotec, Raleigh USA) following manufacturer's protocol. Briefly; anti-bovine IL-4 coating mAb diluted 1:500 in kit coating buffer was pipetted into flat bottomed 96 well ELISA plates, 100µl per well and incubated at 4^{o}C overnight. The following day plates were removed from the refrigerator and contents removed and plates washed three times in wash buffer consisting of 0.2M NaCl with 0.05% Tween-20. A 4% BSA in PBS blocking was then added, 200µl per well, and incubated at room temperature for 1 hour. Following incubation with blocking buffer, wells were emptied of contents and washed as described previously. A freeze dried IL-4 standard was re-constituted in 500µl of distilled water to form stock solution from which standard concentrations were prepared (*APPENDIX B.3*).

Test samples were prepared for incubation, with ConA stimulated culture supernatants diluted 1:2 in wash buffer whilst medium control and SomAg samples were undiluted. Standards and samples were added to the plate in duplicate, 50µl per well. Plates were incubated at room temperature for 1 hour, following which plates were emptied and washed as described

previously. Anti-bovine IL-4 detection mAb was then added at a dilution of 1:500 in wash buffer, 100µl per well, and incubated for 1 hour at room temperature then emptied and washed as previously described. TMB was added, 100µl per well, and incubated in the dark for 30 minutes to allow colour development after which the reaction was stopped with 0.5M HCl, 100µl per well, and absorbance read at 450nm.

To calculate IL-4 concentration, a standard curve was constructed using linear regression analysis on untransformed standard concentration and OD values.

2.9.2.2 IL-4 Sandwich ELISA

A sandwich capture ELISA using paired antibodies as described by Hope et al. (2005) was to quantify IL-4 concentration for the chronic infection study (Chapter 5). This method was used rather than the Serotec ELISA kit (Section 2.9.2.1) used in the primary exposure study since the Serotec kit was not available for purchase when running these samples.

Ninety-six well Nunc Maxisorp ELISA plates (Thermo Fisher Scientific, Waltham USA) were coated with 100µl per well of mouse anti-bovine Interleukin-4 mAb CC314 at 6µg/ml, diluted in distilled water. Plates were incubated overnight at room temperature. The following day, plates were emptied of contents and washed five times with PBS with 0.05% Tween-20 wash buffer (*APPENDIX B.5*). Plates were loaded with 0.1% casein blocking buffer (*APPENDIX B.5*), 200µl per well, for 1 hour at room temperature, following which plates were emptied and washed as before previously described.

Recombinant bovine IL-4 standard was prepared as described by Hope et al. (2005); serial 1:3 dilutions were made in blocking buffer from stock solution (8000pg/ml) (table 2.3). Samples were added to a mixing plate at a 1:2 dilution in blocking buffer for ConA stimulated culture supernatants, whilst medium control and SomAg stimulated culture supernatants were added undiluted. Standards and samples were then added to the plate in duplicate, 100µl per well, and incubated for 1 hour at room temperature before washing plates as previously described.

Plates were incubated with the biotinylated mouse anti-bovine Interleukin-4 mAb CC313 at 2µg/ml, 100µl per well, diluted in blocking buffer for 1 hour at room temperature then washed as previously described. Plates were then incubated with of Streptavidin-HRP (GE Healthcare, Amersham UK) at a dilution of 1:500 in blocking buffer, 100µl per well, for 45 minutes at room temperature before repeating the wash step as previously described. Plates were then incubated at room temperature in the dark with TMB, 100µl per well, for 10-15 minutes to allow colour development after which the reaction was stopped with 0.5M HCl at a volume of 100µl per well and absorbance read at 450nm.

Table 2.3: Standard dilutions for IL-4 sandwich ELISA

Standard ID	Concentration (pg/ml)	Composition (from 8000pg/ml standard)
A	750	40µl rIL-4 standard + 386.6µl blocking buffer
B	250	150µl standard A + 300µl blocking buffer
C	83.33	150µl standard B + 300µl blocking buffer
D	27.78	150µl standard C + 300µl blocking buffer
E	9.26	150µl standard D + 300µl blocking buffer
F	3.09	150µl standard E + 300µl blocking buffer
G	1.03	150µl standard F + 300µl blocking buffer
H	0.34	150µl standard G + 300µl blocking buffer

To calculate IL-4 concentration, a standard curve was constructed using linear regression analysis on untransformed standard concentration and OD values. Both mouse anti-bovine IL-4 monoclonal antibodies (CC313 & CC314) and recombinant IL-4 standard were kindly provided by Dr Jayne Hope, Roslin Institute, Edinburgh.

2.9.3 Interleukin-10

A sandwich capture ELISA using paired antibodies as described by Kwong et al. (2002) to quantify IL-10 concentration from PBMC culture supernatants (Section 2.8.2). For these assays, ConA samples were not included.

Ninety-six well Maxisorp ELISA plates were coated with mouse anti-bovine Interleukin-10 mAb CC318 (Serotec, Raleigh USA) at 6µg/ml, diluted in distilled water, 100µl per well. Plates were incubated overnight at room temperature. The following day, plates were emptied and washed times with PBS with 0.05% Tween-20 wash buffer. Plates were loaded with 0.1% casein blocking buffer, 200µl per well, for 1 hour at room temperature. At the completion of the blocking step plates were emptied and washed as described previously. Recombinant bovine IL-10 standards (kindly provided by Dr Jayne Hope, Roslin Institute, Edinburgh) was prepared as described by Kwong et al. (2002); serial 1:3 dilutions in blocking buffer were made from stock solution (3000ug/ml) as detailed in (table 2.4). Standards and undiluted samples were added to the plate in duplicate, 100µl per well and incubated for 1 hour at room temperature before washing as previously described.

Plates were incubated with biotinylated mouse anti-bovine Interleukin-10 mAb CC320 (Serotec, Raleigh USA) at 2µg/ml, 100µl per well, diluted in blocking buffer for 1 hour at room temperature before washing as described previously. Plates were incubated with Streptavidin-HRP (GE Healthcare, Amersham UK) diluted 1:500 in blocking buffer, 100µl per well, for 45 minutes at room temperature then washed as previously described. Plates were incubated at room temperature with TMB, 100µl per well, for 10-15 minutes in the dark to allow colour development after which the reaction was stopped with 0.5M HCl, 100µl per well, and absorbance read at 450nm.

Table 2.4: Standard dilutions for IL-10 sandwich ELISA

Standard ID	Concentration (IU)	Composition (from 3000u/ml standard)
A	900	120µl stock rIL-10 + 280µl blocking buffer
B	300	150µl standard A + 300µl blocking buffer
C	100	150µl standard B + 300µl blocking buffer
D	33.33	150µl standard C + 300µl blocking buffer
E	11.11	150µl standard D + 300µl blocking buffer
F	3.70	150µl standard E + 300µl blocking buffer
G	1.23	150µl standard F + 300µl blocking buffer
H	0	300µl blocking buffer

To calculate sample concentration, standard OD and concentration values were log transformed and point to point regression performed as for the IFN-γ ELISA (Section 2.9.1).

2.9.4 Transforming Growth Factor-β ELISA

To analyse TGF-β concentration from PBMC culture supernatants (Section 2.8.2), a commercially available TGF-β_1 E_{max}® ImmunoAssay System (Promega, Madison USA) was used. Standards and stock solutions were all stored at -20^{o}C. Prior to assays being performed all required materials were removed from storage and allowed to come to room temperature. This kit is designed for the detection of human TGF-β from a variety of sources including plasma, cell culture and it has been shown previously to detect bovine TGF-β (Abbott et al., 2005). Samples were acid-treated to convert TGF-β to bioactive form detectable by the ELISA. Samples were initially diluted to 1:16 in distilled water as per manufacturer's recommendations for FCS containing samples (Promega Technical Bulletin TB196) and acid treatment performed following the procedure outlined by Abbott et al. (2005); samples were acidified by adding 1N HCl to samples at a volume of 1µl HCl per 50µl of sample, mixed and 1µl taken to check for a pH <3 with universal indicator paper (Camlab, Cambridge UK). Following 15 minute incubation at room temperature samples were neutralised with 1N NaOH, 1µl per 50µl of sample, mixed, and checked for a pH ~7.6 with universal indicator paper. Following dilution and acid treatment, samples were refrigerated at 4^{o}C and analysed the following day. As was the case with the IL-10 ELISA (Section 2.9.3) ConA samples were not included.

ELISA protocol was carried out following the manufacturer's recommendations (Promega Technical Bulletin TB196); Briefly 96 well Maxisorp ELISA plates were coated with anti TGF-β_1 monoclonal coating antibody diluted 1:1000 in 0.025M carbonate coating buffer (*APPENDIX B.7*), 100µl per well and refrigerated overnight at 4^{o}C. The following day, plates were emptied of contents and blocking buffer was added to plates, 270µl per well, and incubated for 35 minutes at 37^{o}C. Following completion of the incubation part of the blocking step, plates were removed from the incubator, emptied and washed five times in TBST (*APPENDIX B.8*). Standards were prepared by serial dilution from a stock solution of TGF-β (1µg/ml) using serial dilutions to produce 7 standard dilutions ranging from 1000 - 15.6pg/ml and a blank standard

of dilution buffer. Standards and samples were added to the plate in duplicate, 100µl per well, and incubated at room temperature for 90 minutes. Plates were washed as described previously, following which secondary anti TGF-β_1 polyclonal detection antibody was added at a 1:1000 dilution in sample dilution buffer, 100µl per well and incubated on a plate shaker for 2 hours at room temperature. Plates were washed as described previously and TGFβ-HRP conjugate was added at a 1:100 dilution in sample dilution buffer, 100µl per well and incubated on a plate shaker for a further 2 hours at room temperature. Plates were then washed as described previously and TMB was added, 100µl per well and plates incubated for a further 15 minutes in the dark at room temperature after which the reaction was stopped with 0.5M HCl and absorbance read at 450nm.

Standard curves were constructed using untransformed sample concentration and OD values with simple linear regression analysis.

2.10 Real-time quantitative PCR

2.10.1 RNA Extraction and Quantification

RNA extraction was carried out using the commercially available RNeasy Mini Kit (QIAGEN, Limburg Netherlands) following manufacturer's recommendations. Samples in RNAlater (Section 2.8.2) were thawed on ice, centrifuged at 5000g to pellet PBMCs and supernatants discarded. Following this, 350µl of "RLT" buffer was added to the cell pellet and vortexed to disrupt and homogenize PBMCs, then 350µl of 70% ethanol was added and mixed by pipetting. The total sample volume (700µl) was transferred to an RNeasy Mini spin column placed in a 2ml collection tube, the lid was closed and samples centrifuged for 15 seconds at 8000g. Flow-through was discarded, and 700µl "RW1" buffer was added to the RNeasy spin column. Samples were then centrifuged for a further 15 seconds at 8000g. Flow-through was again discarded and 500µl "RPE" buffer was added to the RNeasy spin column. Samples were centrifuged for a further 15 seconds at 8000g. The "RPE" wash step was repeated, after which RNeasy spin columns were placed in fresh eppendorfs and centrifuged at 8000g for 1 minute to the dry membrane after which 40µl Ambion® RNase-free water (Thermo Fisher Scientific, Waltham USA) was added directly to the column membrane and centrifuged for 1 minute at 8000g to elute the RNA.

RNA concentration was quantified using ribogreen kit (Invitrogen Life Technologies, Grand Island USA). Standards of known concentration of template RNA prepared from stock by serial dilutions in working concentration "TE" buffer to produce a standard curve range of 1000-20ng/ml. Samples were diluted in "TE" buffer to give a final 1:500 dilution for quantification. Standards and samples were pipetted into fluorescence micro plate strips, 100µl per well, and incubated in the dark for 5-10 minutes. Fluorescence was measured based on 485nm (±20nm)

excitation and 535nm (±25nm) emission. Sample concentrations were calculated from linear regression of untransformed emission data from standards.

2.10.2 cDNA Synthesis

cDNA was synthesized using the commercially available Quantitect Reverse transcriptase kit (QIAGEN, Limburg Netherlands) following manufacturer's recommendations. Kit reagents were removed from storage at -20°C ahead of use and allowed to thaw at room temperature. Prior to cDNA synthesis, a DNA digestion step was performed to remove unwanted genomic DNA potentially retained from the RNA extraction; 1µg sample RNA was diluted in RNase free water to a total volume of 12µl, to which 2µl of "gDNA wipeout" buffer was added. Samples were placed on a heat block and incubated for 2 minutes at 42°C before being placed immediately back on ice. Following this, cDNA master mix consisting of 1µl Quantiscript Reverse Transcriptase, 4µl Quantiscript RT Buffer and 1µl RT Primer mix was prepared per reaction. Six microliters of this master mix was then added to each sample to make a total reaction volume of 20µl and mixed. Samples were incubated at 42°C for 15 minutes, then 95°C for 3 minutes to inactivate the Quantiscript Reverse Transcriptase, after which samples were stored at -20°C ahead of qPCR.

2.10.3 SYBR green qPCR

Bovine cytokine transcripts for interleukin-2 and interleukin-5 were quantified by qPCR and normalised against 28s ribosomal RNA as previously described (Hansen et al., 2011; Rosbottom et al., 2007).

Primers for interleukin-2 and 28s were taken from previous studies (Rosbottom et al., 2008; Rosbottom et al., 2007), whilst primers for interleukin-5 were taken from Freeman et al. (2008) (table 2.5). Quantitative qPCR using SYBR Green was performed as described by Hansen et al. (2011). Primers were reconstituted to 100µM in DNase free water according to manufacturer's specifications (Eurofins MWG, Ebersberg Germany), and further diluted to a working stock solution of 4µM, aliquoted and stored at -20°C ahead of use.

Master mix was prepared at a volume of 19µl per reaction, containing 10µl of 2× SensiMix with SYBR Green I and primers at concentrations specified (table 2.6), with the remainder of the 19µl volume made up with DNase free water, to which 1µl of cDNA template was added, making a total volume of 20µl per reaction.

Samples were assayed alongside known concentration standards consisting of either plasmids into which the full-length cDNA was cloned, as was the case for 28s and IL-2 (Rosbottom et al., 2008; Rosbottom et al., 2007), or for IL-5 of a synthetic oligonucleotide representing the full-length amplicon (Hansen et al., 2011). All standards were diluted in a 10-fold series in 100ng/µl

yeast tRNA (Invitrogen Life Technologies, Grand Island USA) to prevent aggregation. Standard curves of these reagents ($5 \times 10^6 – 5 \times 10^{-1}$ copies) were included in duplicate on every plate. Reactions were performed on a DNA Engine opticon 2 continuous Fluorescence detector under the following conditions: 10 min at 95°C for initial denaturation; 35-40 cycles as specified (table 2.6) at 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 15 seconds for PCR amplification; melting curve analysis was then performed between 50 and 95°C to confirm the specificity of the amplification products. Ct cut-off values were adjusted to the log-linear phase of amplification following which sample concentrations were determined using a linear regression model based on the concentrations of the standards. For 28s, where different dilutions yielded significantly different values, the 1:1000 dilution was used for consistency and reduce the risk of sample inhibition in higher concentrations.

Table 2.5: qPCR primer sequences for bovine 28s and cytokine expression

Target gene	Direction	Primer sequence (5' to 3')	NCBI accession No.	Reference
28s	Forward	GGCGAAAGACTAATCGAACCA (21)	AF154866	(Rosbottom et al., 2007)
	Reverse	CGAGAGCGCCAGCTATCCT (19)		
IL-2	Forward	GGAGAAAGTTAAAAATCCTGAGAACCT (27)	M12791	(Rosbottom et al., 2008)
	Reverse	TTCTAGTAAACACTTAAGATGTTTCAATTCTG (32)		
IL-5	Forward	TGGCAGAGACCTTGACACTG (20)	EU915048.1	(Freeman et al., 2008)
	Reverse	TGGTGATTTGTATGCTGAGGA (21)		

2.11 Peripheral Blood Leukocyte Enumeration

The enumeration of peripheral blood leucocytes was determined using a combination of techniques.

2.11.1 Haematology

Total leukocyte counts in peripheral blood were determined using a 102 FastRead™ multi chamber haemocytometer following a 1:20 dilution of EDTA treated whole blood in 2% Acetic acid with 1% gentian violet (*APPENDIX B.9*) to haemolyse erythrocytes and to stain the nuclei of remaining leucocytes.

Table 2.6: qPCR reaction details for bovine cDNA sequences

Target gene	Primer concentration (µM)	Sample dilution	Number of PCR cycles	Amplicon size (bases)
28s	0.2 (1ul)	1:100, 1:1000	35	72
IL-2	0.6 (3ul)	1:10	40	112
IL-5	0.2 (1ul)	1:10	35	94

Differential cell counts were carried out on thin blood films prepared with Wright's stain following manufacturer's recommendations (Procedure No. WS, Sigma-Aldrich, St. Louis USA). Once prepared, smears were examined at the tapered edge under oil immersion at x100 magnification. Differential counts were carried out on a minimum of 100 cells with identification based on the morphological characteristics (figure 2.3). Damaged and/or morphologically indeterminate cells were excluded from counts. Peripheral blood counts for each cell phenotype were then calculated as a percentage of total leukocyte counts.

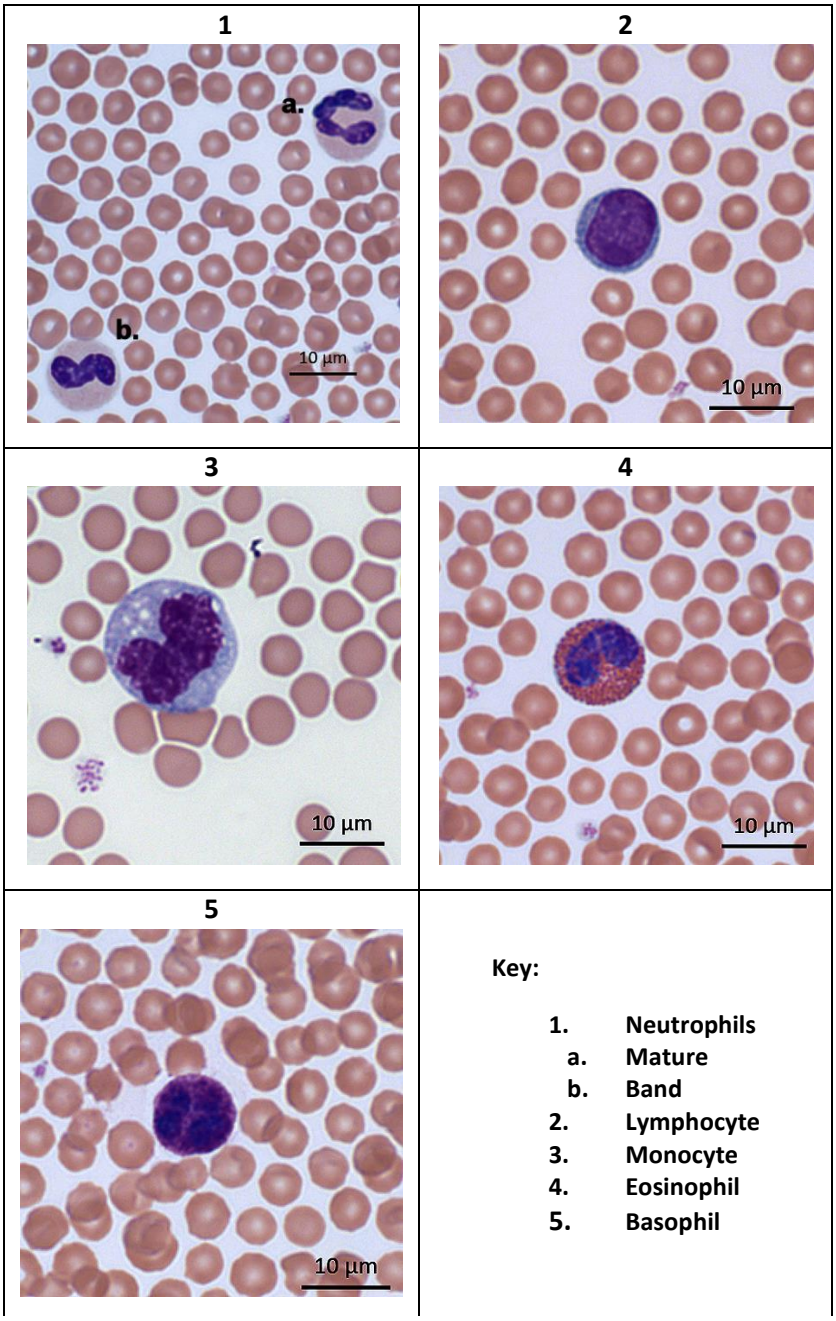

Figure 2.3: Leucocyte appearance with Wright's stain

2.11.2 Flow Cytometric PBMC Analysis

2.11.2.1 One Colour Indirect Immunofluorescence Labelling

PBMCs were adjusted to a concentration to 1 x 10^7 cells/ml in PBS/EDTA and pipetted out in duplicate onto a 96 well round bottomed plate, 5 x 10^5 cells per well (50µl per well).

Cells were pelleted by centrifuging the plate for 5 minutes at 500g, after which supernatant was removed and 25µl of mAb diluted as specified (table 2.7) in PBS containing 1% BSA and 0.1% sodium azide (PBS/BSA/Az) was added to each sample in duplicate and cells re-suspended and incubated for 10 minutes. Following this, samples were washed with PBS/BSA/Az, 100µl per well, and centrifuging for 5 minutes at 500g, after which wells were emptied by brisk flicking, and the wash step was repeated.

Table 2.7: Primary monoclonal antibody dilutions for indirect immunofluorescence staining of PBMCs

Antibody	Cell surface receptor	Dilution
IL-A11	CD4	1:10
CC-63 (Serotec MCA837GA)	CD8	1:100
CC-15	WC1	1:10
CCG-33	CD14	1:10

For detection, 25µl of FITC-conjugated Goat anti-mouse IgG (Sigma-Aldrich, St. Louis USA) diluted 1:200 in PBS/BSA/Az was added to all samples with the exception of blanks and incubated in the dark on ice for 10 minutes, then washed twice and fixed by adding PBS/BSA/Az/containing 1% neutral buffered formalin, 100µl per well. Fixation was achieved through incubation for 10 minutes on ice in the dark, ahead of two further washes, after which samples were stored in 200µl of PBS/0.1% BSA at 4^{o}C before FACS analysis. All incubation steps were performed on ice.

2.11.2.2 Gating Strategy for PBMC Analysis

Samples were run through the MACSQuant® Analyser using a standardised running buffer (Miltenyi Biotec, Bergisch Gladbach, Germany), the majority of PBMCs were clustered in a distinct population of moderate forward scatter, with a low level of side scatter (figure 2.4).

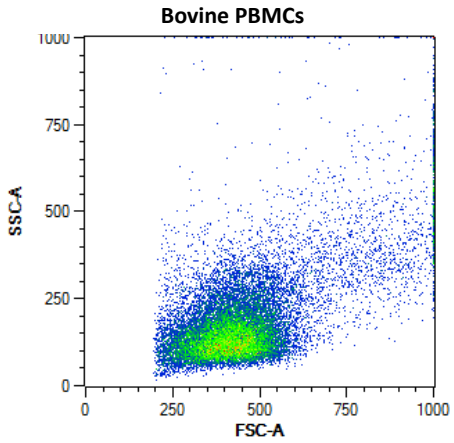

Figure 2.4: PBMCs populations under forward and side scatter analysis

It was noted that the majority of $CD14^+$ PBMCs lay adjacent to the main cell population. This was to be expected since these cells are on the whole larger and less uniform, with $WC1^+$ cells appearing to be an 'intermediary' population in terms of side scatter. There was considerably less variation observed between these populations in terms of forward-scatter, although both $CD14^+$ and $WC1^+$ populations had a greater level of forward scatter than $CD4/8^+$ populations.

As a consequence, an analysis template was devised for which $CD4/8^+$ PBMCs would be analysed through gating the area of greatest PBMC concentration, whilst a wider gate would be used to analyse $WC1^+$ and $CD14^+$ PBMCs, to take into account these cells having greater FSC and SSC (figure 2.5).

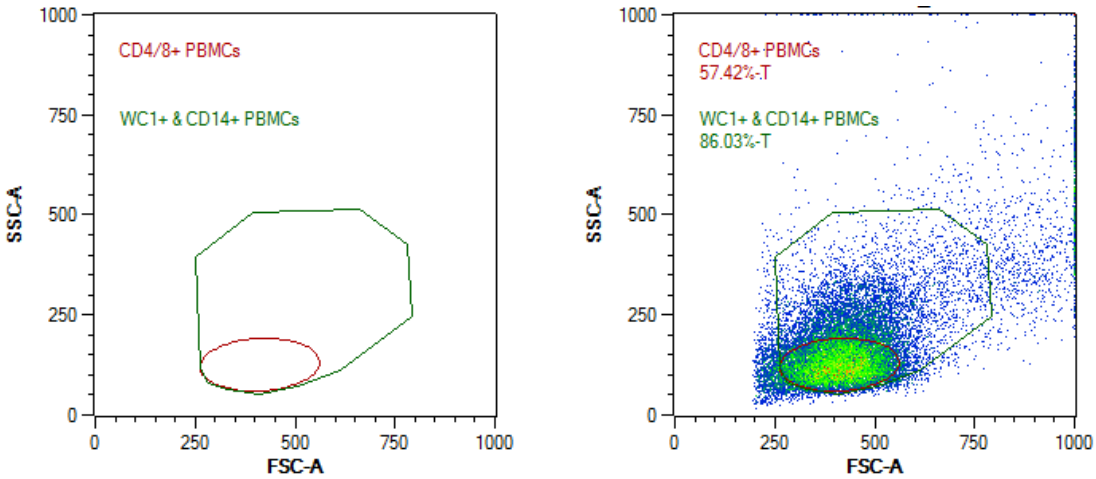

Figure 2.5: Gating strategy for CD4/8+ PBMCs vs. CD14+/WC1+ PBMCs based on FSC/SSC

From these templates we determined the percentage populations; the gated populations described were analysed for FITC-fluorescence using the appropriate excitation/emission channel (488nm/500-550nm), assessed using a frequency histogram and gating to select FITC-positive cells (figure 2.6).

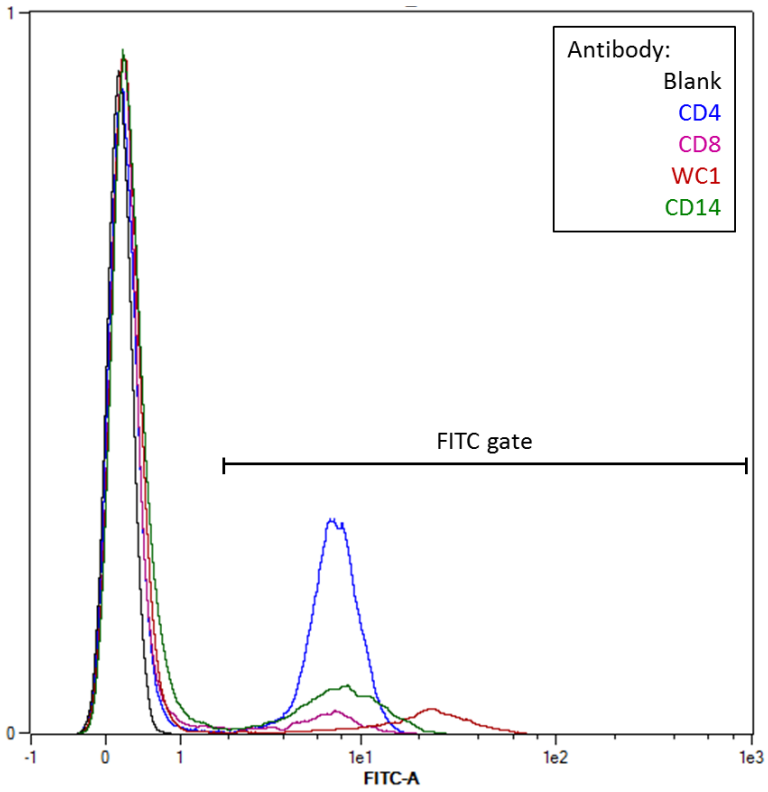

Figure 2.6: Gating for FITC-positive PBMCs and representative phenotypes

FITC control blanks were subtracted from each mAb positive sample analysis to compensate for auto fluorescence, although typically this did not exceed 0.1%. Absolute cell counts in peripheral blood were then calculated as a percentage of the total lymphocyte counts determined via haematology (Section 2. 11.1).

Chapter 3

Evaluation of a Composite Faecal Egg Count Technique for the Diagnosis of *Fasciola hepatica* in Groups of Cattle

3.1 Abstract

A method to identify liver fluke (*F. hepatica*) eggs in groups of cattle from composite samples, made using 5g from each of ten individual cows was assessed through comparison with counts from individual sedimentation analysis and group level prevalence. Individual egg count data (n=638) for groups of animals from 29 farms was fitted to Negative Binomial Distributions (NBDs) to generate predicted composite counts through stochastic re-sampling. The resulting data was compared to observed composite counts (n=14) and summed individual counts (n=59) using multivariate linear regression and the sensitivity calculated relative to individual egg count analysis. Our results showed a 95% sensitivity for composite counts at ≥0.4 eggs per gram of faeces, indicating that using a composite sample analysis is as sensitive as performing counts on ten individual samples. In addition, a strong positive correlation was shown between composite egg counts and both summed individual egg counts and group prevalence. These findings suggest fluke egg counts obtained from composites represent a valuable diagnostic tool for the detection of *F. hepatica* infection in groups of cattle, particularly beef and non-lactating dairy cattle.

3.2 Introduction

The digenean trematode *Fasciola hepatica* is an important parasite of livestock in the UK, predominantly infecting sheep and cattle. Both clinical disease (fasciolosis) and sub-clinical infections represent a major source of economic loss through morbidity, mortality and impaired productivity. Sub-clinical infection leads to reduced growth or weight gain, fertility and reduced milk yield (Charlier et al., 2007; Howell et al., 2015; Lopez-Diaz et al., 1998). Data from 2010 show liver condemnations in 22% of all bovine carcasses processed (>2.2 million) in England, Scotland and Wales at an estimated cost of £3.2 million (EBLEX, 2011). Disease surveillance shows increasing prevalence of fasciolosis in recent years both in endemic regions, and in areas where *F. hepatica* has not previously been considered of major importance (Kenyon et al., 2009; Pritchard et al., 2005). Much of this can be attributed to climate, with ambient temperatures between 10-30^{o}C and high levels of rainfall and moisture favouring the development of both the external life stages of *F. hepatica* and its intermediate snail host, *Galba truncatula* (Torgerson and Claxton, 1999). This link between changing weather patterns and increasing prevalence has been further demonstrated through climate-driven models

which show a clear association between *F. hepatica* infection and both ambient temperature and moisture levels in the UK and Europe (Caminade et al., 2015; Fox et al., 2011). Furthermore, these models suggest that with projected changes in weather patterns the spatial and temporal ranges of *F. hepatica* will increase further in the coming years and decades.

Control of *F. hepatica* is reliant upon a limited number of anthelmintics with activity against *F. hepatica* (flukicides), of which triclabendazole (TCBZ) is the most commonly used due to its high level of efficacy against both juvenile and adult stages of the parasite (Boray et al., 1983). This has led to the emergence of drug resistance in the UK and abroad (Gordon et al., 2012; Moll et al., 2000; Ortiz et al., 2013; Overend and Bowen, 1995). Alternative control methods such as vaccination remain several years away from commercial availability (Molina-Hernandez et al., 2015; Toet et al., 2014), meaning there is a need to re-evaluate how current control methods, anthelmintics especially, are implemented in order to use them in an effective yet sustainable manner (COWS, 2013).

Increasing the use of diagnostics to inform treatment will help to reduce inappropriate use of anthelmintics and reduce selection for resistance. Three methods are used routinely in the UK to diagnose fluke infection in cattle; fluke egg counts, a *F. hepatica*-specific antibody ELISA and a copro-antigen (CoAg) sandwich ELISA.

There are several methods used to detect fluke eggs in faeces, ranging from sedimentation techniques through to methods that involve centrifugation and/or flotation following initial sieving of the sample (Anon., 2007; Charlier et al., 2008b; Cringoli et al., 2010; Taylor et al., 2016). Eggs are easily identifiable under low power microscopy (4-10x); they are 130-145 x 70-90µm and ovoid in shape, with a single operculum, typically golden brown in colour and have a refractile appearance. Egg counts, by definition, only detect patent infections, typically from 10 weeks post infection onwards which, combined with the high degree of variation in faecal egg output, particularly in cattle, makes them relatively insensitive. Conversely, specificity is high since where fluke eggs are observed infection is very likely to be present, although eggs can be trapped in the gall bladder and released following TCBZ treatment, potentially leading to false positives (Toner et al., 2011). Sedimentation is the most widely used method for fluke egg counts due to its relative simplicity, and since it allows the use of relatively large quantities of faecal material, which has been shown to be increase test sensitivity; Rapsch et al. (2006) demonstrated that sedimentation using 30g of faeces gave a sensitivity of 91.9% compared with 69% when 10g of faeces were used.

Enzyme-Linked Immunosorbent Assays have been developed to detect bovine IgG antibody to *F. hepatica* Excretory/Secretory (E/S) antigen in both serum and milk samples (Salimi-Bejestani et al., 2005a). These responses have been shown to be present in experimentally infected

cattle from 2-4 weeks post infection, with antibody titre shown to correlate with intensity of infection at an overall quoted test sensitivity and specificity of 98% and 96%, respectively (Salimi-Bejestani et al., 2008; Salimi-Bejestani et al., 2005b). In addition to diagnosing infection in individual animals, the E/S antibody ELISA can also be performed on Bulk Milk Tank (BMT) samples to identify *F. hepatica* infection in lactating dairy herds. BMT antibody values have been shown to correlate with herd level prevalence as measured by both serum antibody and worm egg count with a test sensitivity and specificity of 96% and 80% for BMT analysis, respectively when compared to individual serum sample analysis (Salimi-Bejestani et al., 2005a).

The CoAg ELISA is a commercially available sandwich ELISA that detects *F. hepatica* specific E/S antigen in faeces. It detected infection from 5 weeks post infection in experimentally infected lambs (n=50), with a 100% sensitivity from 10 weeks post infection and 93% in naturally infected cattle (n=80) when compared to post-mortem examination (Mezo et al., 2004).

Direct comparison of these three diagnostic tests (worm egg counts, E/S antibody ELISA and CoAg ELISA) to post-mortem finding in adult cattle (n=200) found that faecal egg counts had the lowest sensitivity but highest overall specificity, whilst the CoAg was found to be the most sensitive (Charlier et al., 2008b). Conversely, Duscher et al. (2011) reported a relative sensitivity of only 30% for the CoAg ELISA when compared to serum antibody ELISA in naturally infected cattle (n=595) and a relative sensitivity of 40% for fluke egg counts when using a standard sedimentation technique. These data suggest that under field conditions the sensitivity of the CoAg ELISA may be more similar to fluke egg counts.

In terms of practicality, both the antibody and CoAg ELISAs have distinct advantages over faecal egg counts, allowing multiple samples to be processed and tested rapidly. However the need to send samples to a dedicated diagnostics laboratory with added time and cost implications may deter farmers and clinicians from using these options. Conversely fluke egg count by sedimentation can be performed in house by either clinicians or trained staff and, aside from purchase of initial equipment such as test sieves and a low power microscope, are inexpensive. Although they lack sensitivity, if positive they provide a definitive diagnosis provided treatment history is known.

All three methods have been validated for use in individual animals. However, currently only the BMT antibody ELISA has been validated as a method for identifying infection in groups of cattle. This is a useful screening tool, as it allows monitoring of herd level infection and the efficacy of control programs in lactating cattle, indicating when further action such as individual diagnoses and/or treatment is warranted (Salimi-Bejestani et al., 2005a). Currently herd level screening in beef and non-lactating dairy cattle is reliant upon sampling multiple individual animals and testing them for infection, which is both time consuming and expensive.

Hence there is a need for a simple, rapid screen to allow the rapid identification of fluke infection in groups of animals, such as beef cattle, to allow the identification of infection within a herd and evaluate the efficacy of control programmes. Fluke egg counts using composite faecal samples have been shown to be an effective way of identifying presence of infection in groups of sheep (Daniel et al., 2012). In this chapter, we assess the sensitivity of a composite faecal egg count method compared with individual egg counts and its potential use as a herd/group level diagnostic test for *F. hepatica* infection in cattle.

3.3 **Materials and Methods**

3.3.1 Sample Collection

Faecal samples (n=138) were collected from 7 beef herds (A.1 to A.7) with an average herd size of 86 animals (32-230) in the region surrounding Bala, Gwynedd, north-Wales. Twenty individual samples were collected per farm. To reduce the likelihood of re-sampling the same animal, fresh samples were collected by walking in a zig-zag pattern across the pasture where animals were currently gazing. Samples were transported to the laboratory in sealed plastic containers and stored at 4^{o}C until processing to prevent development of *F. hepatica* eggs (Rowcliffe and Ollerenshaw, 1960). Additional fluke egg count data taken from a previous study (farms B.1 to B.22) were used as part of the statistical analysis (Section 3.3.3).

3.3.2 Fluke Egg Sedimentation

Fluke egg counts were performed on both individual and composite samples using a standard sedimentation technique (Anon., 2007). For individual counts, 10g of faeces was used, whilst for composite counts, 5g of faeces were taken from ten individual samples and combined to give a 50g composite sample. Two composite samples were processed and analysed per farm (A.1 to A.7), giving 14 composite egg counts in total.

Faeces were homogenised in tap water and passed through stacked sieves (Endecotts Ltd., London England) of 750µm, 150µm and 38µm mesh sizes respectively, with the largest mesh sieve (750µm) on top and the smallest (38µm) at the bottom. Samples were washed through the sieves with copious volumes of tap water to break up un-fragmented material. Once the water running from the bottom sieve was clear, the top two sieves were removed and their contents discarded. The retentate on the 38µm sieve was transferred to a glass beaker and diluted to a volume of 500ml with tap water. The suspension was left to stand for 4 minutes to allow sedimentation of *F. hepatica* eggs following which the supernatant was decanted and the beaker re-filled to 500ml with water. This process was repeated until no suspension remained after the 4 minute sedimentation. The supernatant was then decanted and the

sediment transferred either directly to a petri-dish for microscopy, or to a 50ml falcon tube (VWR, Radnor USA) for storage at 4^{o}C.

Samples were counted using a stereo dissecting microscope at 4-10x magnification. To aid counting, methylene blue was added to the sample to stain background material. For samples with greater quantities of sediment, the sample divided between 2-3 petri-dishes to ensure accurate counting.

3.3.3 Statistical Analysis

Composite egg counts for farms A.1 to A.7 were compared to the sum of counts from the individual sample analysis using standard linear regression to determine whether composite counts were what would be expected based on individual sample analysis. To achieve this, individual egg counts for each of the ten faecal samples used to make each composite sample were added together and divided by 2 to give a sum of individual counts per 50g faeces (individual counts were performed on 10g faeces).

The relationship between composite and individual counts was further investigate by fitting Negative Binomial Distributions (NBDs) to the individual egg count data for each farm (A.1 to A.7) by 'Maximum Likelihoods' (ML) using free online statistics software to give a k-value describing the overdispersion (skew) of the population distribution (Wessa, 2014). The fitted k-values and mean (μ) egg count for each population were then used to generate NBDs using the Pop Tools Add-in for Microsoft Excel from which repeated random sampling could be performed. These NBDs were checked against the original count data for goodness of fit using a X^2 test. Fitted k-values, arithmetic mean and goodness of fit for the individual worm egg count data for farms A.1 to A.7 are summarised in table 3.1.

Table 3.1: **Parameters used to generate Negative Binomial Distributions (NBDs) for farms A.1 to A.7.** k-value indicates overdispersion (skew) and μ the mean egg count for each fitted NBD. X^2 p-values are the result of comparison between original fluke egg counts and NBD-predicted fluke egg counts.

Farm ID	k-value	Mean (μ)	X^2 p-value
A.1	1.048	14.750	0.194
A.2	0.914	13.850	0.297
A.3	3.905	10.150	0.276
A.4	0.594	0.400	0.878
A.5	2.053	48.667	0.262
A.6	2.152	47.550	0.253
A.7	0.488	2.500	0.878

In addition to the individual counts from farms A.1 to A.7, individual egg count data collected as part of a previous investigation were also used for this part of the analysis to increase overall sample size and thus improve model fit (Salimi-Bejestani et al., 2005a). These counts were based on 1g individual faecal samples (n=500) collected from 22 UK dairy farms (B.1 to B.22). Twenty to 33 samples were collected per farm, representing 20% of the total milking

herd. NBDs were fitted to the individual count data for farms B.1 to B.22 and assessed for goodness of fit as described above (*APPENDIX C*). Since these individual counts were performed on 1g of faeces as opposed to the 10g used for farms A.1 to A.7, the inclusion of this data also allowed us to consider how different quantities of faeces impacted on sensitivity. Using predicted individual egg counts generated from the fitted NBDs for each farm (n=29) NBD-predicted composite counts per 50g of faeces were calculated in the same way that the sum of the observed individual counts had been calculated previously; for counts derived from 10g faecal samples (A.1 to A.7), ten individual counts were added together and divided by 2, whilst for counts derived from 1g faecal samples (B.1 to B.22) ten individual counts were added together and multiplied by 5. The NBD-predicted composite counts were randomly generated through stochastic re-sampling of each fitted NBD, using 10,000 iterations per farm, to create a mean NBD-predicted composite count (x_1) and standard deviation (σ) for each farm. From this, a single value, the coefficient of variation (*CV*) was calculated for each farm:

Equation 1:

$$CV = \frac{\sigma}{x_1}$$

Using multivariate linear regression, *CV* was fitted as a response variable (*Y*) against both mean NBD-predicted composite count (x_1) and the quantity of faeces used in grams (x_2) as explanatory variables using data from all 29 farms. Log-transformation was performed on both response and explanatory variables to ensure linear fit:

Equation 2:

$$\log(Y) = \alpha + \beta_1(\log(x_1 + 1)) + \beta_2(\log(x_2))$$

An additional interaction term was considered between quantity of faeces and cumulative egg count, but was not found to improve model fit and was consequently excluded from final model fit (data not shown). The resulting intercept value (α) and slope values (β_n) for each explanatory variable (x_n) were then used to define the standard deviation of the NBD-predicted composite egg count (σ):

Equation 3:

$$\sigma = x_1 \times e^{\alpha+\beta_1(\log(x_1+1))+\beta_2(\log(x_2))}$$

The exponential function (*e*) in *equation 3* represents *CV* (the right side of *equation 2*), meaning this is simply a re-arrangement of *equation 1* where σ has been made the subject and *CV* has been expressed in terms of mean NBD-predicted composite count (x_1) and quantity of faeces (x_2). Using *equation 3* it was therefore possible to calculate standard deviation (σ), and therefore generate 95% confidence intervals. Model generated confidence intervals were checked against the sum of the observed individual counts (per 50g) for farms B.1 to B.22 (*APPENDIX C*) and both observed and summed individual counts (per 50g) for farms A.1 to A.7

(table 3.2), with quantity of faeces (x_2) taken to be 1, 5 and 10, respectively. Finally, the lower confidence interval for 10x5g composite samples (x_2=5) was used to identify the mean NBD-predicted composite egg count (x_1) at which 95% sensitivity was achieved.

As an additional investigation, prevalence of infection for each farm was compared to the observed composite counts using standard linear regression.

3.4 Results

Composite egg counts for farms A.1 to A.7 are shown in table 3.2. These ranged from 0 to 341 eggs per 50g composite sample. Comparison with the sum of individual egg counts (per 50g) showed a close correlation (figure 3.1a), with linear regression analysis giving a slope value (β) of 1.001 (SD ±0.159), intercept (α) of 5.335 (SD ±20.693) and adjusted R^2 of 0.749, indicating observed composite counts are virtually identical to what would be expected based on individual egg counts.

Table 3.2: Summary of composite egg counts for farms A.1 to A.7. Two composite counts and summed individual counts are shown for each farm. NBD-predicted counts show the mean count and 95% confidence intervals for composite counts generated through repeated random sampling of the fitted NBDs described in table 3.1, 10,000 iterations per farm.

ID	Prevalence (%)	Composite egg count (10 x 5g)	Sum of individual counts (50g)	NBD-predicted counts (50g) Mean	Upper 95% CI	Lower 95% CI
A.1	95	96	74	73.706	119.800	27.611
		82	73.5			
A.2	89.474	61	74.5	69.642	116.057	23.227
		46	64			
A.3	100	68	57.5	50.665	69.281	32.048
		57	44			
A.4	25	6	2.5	2.015	4.560	-0.530
		1	1.5			
A.5	100	167	253	243.684	351.277	136.092
		341	185.5			
A.6	100	201	226.5	238.539	341.437	135.641
		269	249			
A.7	52.632	0	11	12.473	24.626	0.319
		12	14			

Individual worm egg count data from farms A.1 to A.7 showed overdispersion, with fitted NBDs showing a close fit to original count data (figure 3.2). No significant difference was found between NBD-generated and observed count data when assessed through X^2 goodness of fit (table 3.1). Similarly, no significant difference was found between NBD-generated and observed count data for farms B.1 to B.22 (*APPENDIX C*).

A positive relationship was observed between the mean NBD-predicted composite count (x_1) and standard deviation (σ). Additionally, standard deviation was also greater in counts where a smaller quantity of faeces (x_2) was used (figure 3.3). When considered in terms of *CV*, log-

transformed values demonstrated a negative linear correlation with NBD-predicted mean counts (x_1), again with a smaller quantity of faeces increasing the overall *CV* (x_2) (figure 3.4).

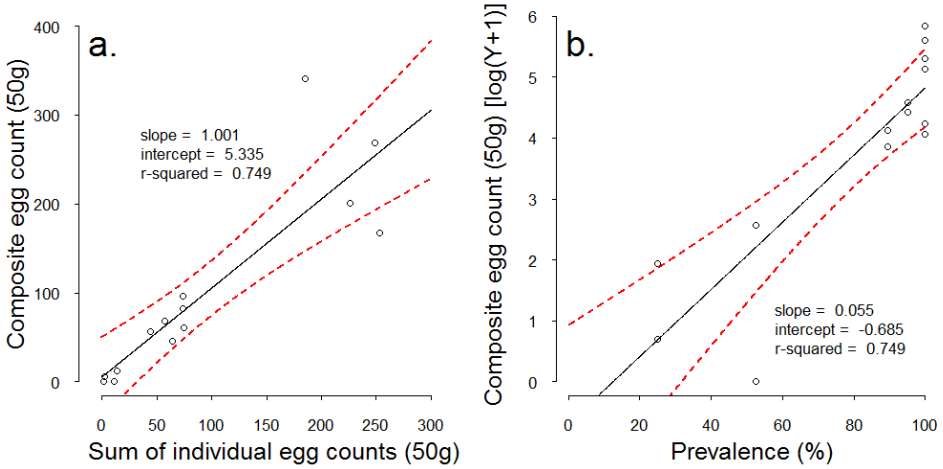

Figure 3.1: (a.) Observed composite egg counts versus sum of the observed individual counts (per 50g) and (b.) logged composite egg counts versus group prevalence. (---) denotes 95% confidence interval for fitted regression lines.

Results of the multivariate linear model analysis of *CV* (*equation 2*) are summarised in table 3.3. Both (logged) NBD-predicted composite counts (x_1) and quantity of faeces (x_2) were significantly negatively correlated ($p<0.001$) with the overall model fit giving an adjusted R^2 of 0.718.

All values for the sum of observed individual egg counts (per 50g) based on both 10g and 1g faecal samples fell within the confidence intervals generated by the model when quantity of faeces (x_2) was taken to be either 10g or 1g (figure 3.5a). Similarly, all observed composite counts from farms A.1 to A.7 also fell within confidence intervals generated by the model when x_2 was taken to be 5g (figure 3.5b).

Table 3.3: Summary of model output (defined in equation 2) using egg count data from all 29 farms with fitted coefficients (slope (β)) and p-value for each explanatory variable (x_n).

Variable	Transformation	Intercept (α)	Coefficient (β_n)	P-value
Response variable (*Y*): Coefficient of variation (*CV*)	log(*Y*)	0.779	-	0.0003
Explanatory variables (x_n):				
NBD predicted FEC (50g)	$\log(x_1 + 1)$	-	-0.342	1.51×10^{-7}
Quantity of faeces (g)	$\log(x_2)$	-	-0.261	0.0004

Where x_2 was taken to be 5g, the lower confidence interval was found to equal zero when mean NBD-predicted composite count was 19.56, meaning a 95% sensitivity at ≥20 eggs per 50g faeces (≥0.4 eggs per gram) suggesting composite sample analysis has a sensitivity similar to that expected from individual sample analysis.

Following log-transformation of composite egg counts, a strong positive correlation was found with prevalence with an adjusted R^2 value of 0.749(figure 3.1b).

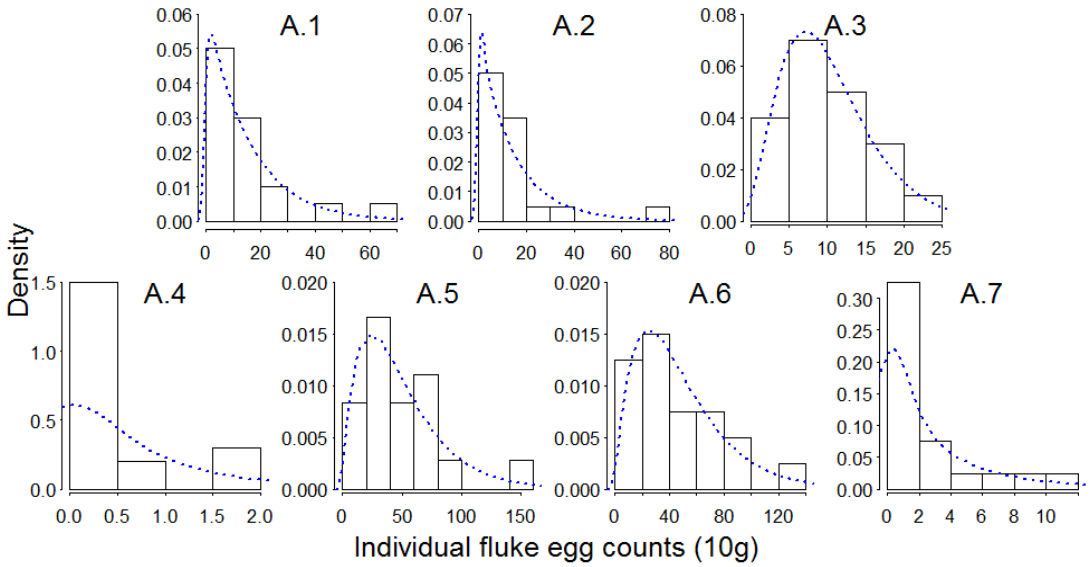

Figure 3.2: Distribution of individual worm egg counts (per 10g faeces) for farms A.1 to A.7 and fitted NBDs. Histograms are displayed as probability density (where sum of the area equals 1) rather than count frequency to allow easier comparison with the fitted NBD (---).

3.5 Discussion

The principle finding of the analysis described in this chapter is that in cattle, egg counts based on a composite of ten, 5g faecal samples has a sensitivity at, or close to, that which would be expected when performing ten individual egg counts on the same quantity of faeces. Regression analysis of composite counts and the sum of observed individual counts (per 50g) showed these two methods to give the same result (i.e. Y = x). Furthermore, the 95% confidence intervals generated from the NBD-predicted composite counts, when quantity of faeces (x_2) was taken to be 5g, indicated that composite sample analysis would detect eggs with 95% certainty at a mean egg count of >19.56, or ≥0.4 eggs per gram of faeces. This analysis demonstrates sensitivity of composite sample counts relative to individual fluke egg counts, which have been shown in cattle to have a sensitivity ranging between 39 and 69% (Charlier et al., 2014; Duscher et al., 2011; Rapsch et al., 2006). It has been shown previously that performing analyses of three serial samples using 10g faecal samples (30g total) increased the sensitivity of the standard sedimentation technique from 69% to 91.9% compared to a single 10g sample analysis (Rapsch et al., 2006). Similarly, a when using a sedimentation-flotation technique, Charlier et al. (2008b) found an increase in sensitivity from 43% to 64% when using 10g instead of 4g sample samples. The results of our model analysis also indicate that increasing the quantity of faecal material used increases overall sensitivity of composite sample analysis in a similar manner. Whilst a 10 x 5g composite sample takes longer than a 10g individual sample to prepare and read, it is much quicker than having to perform ten separate individual counts. Given that our findings suggest using a composite sample has a sensitivity comparable to that of doing 10 individual counts, we conclude that composite faecal egg sedimentation in cattle provides a simple but effective diagnostic test to aid the control of

what is and will continue to be a problematic and complex parasitic disease, particularly since egg sedimentation can be performed in most veterinary practices.

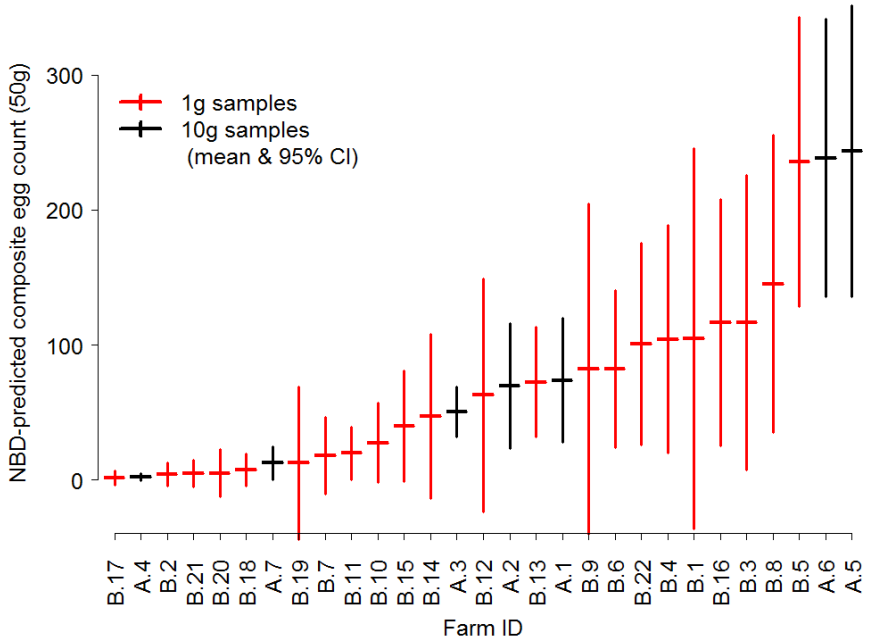

Figure 3.3: NBD-predicted composite egg counts (mean & 95% CI) for each farm (n=29) based on repeated random sampling (10,000 iterations) of NBDs fitted to individual fluke egg count data arranged in order of ascending mean NBD-predicted composite count.

The validity of our model analysis is supported by the observation that all composite counts (n=14) from farms A.1 to A.7 fell within the NBD-generated confidence intervals, as did the sum of observed individual egg counts for all 29 farms, confirming that the final parameterised model was representative of actual observations.

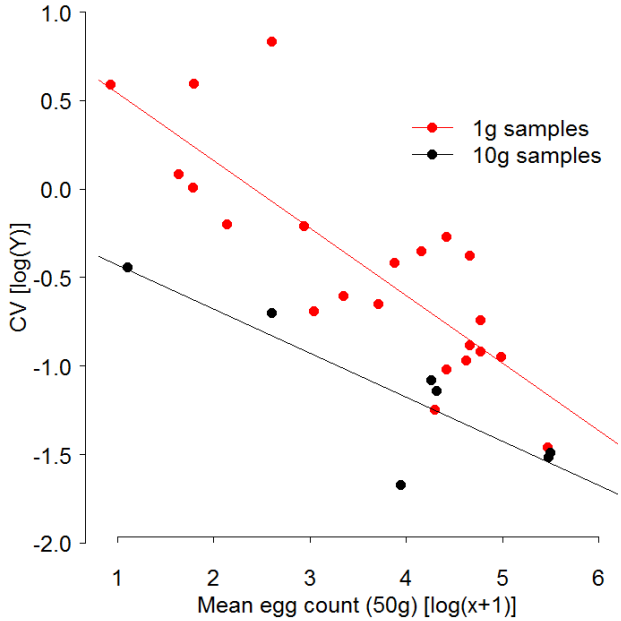

Figure 3.4: (Logged) coefficient of variation (CV) versus (logged) mean NBD-predicted composite egg counts based on individual egg counts from 1g and 10g faecal samples.

Whilst both the summed individual counts and mean NBD-predicted composite counts (x_1) were largely similar to our observed counts it was apparent that the quantity of faeces (x_2)

used to produce these counts affected both the coefficient of variation (*CV*) and standard deviation (σ) of the NBD-predicted composite counts (figures 3.3 & 3.4). This is unsurprising, since increasing the amount of faeces used for fluke egg counts is known to improve test sensitivity (Rapsch et al., 2006). It follows that variation around the mean will reduce since the larger sample will be more representative of the actual egg count. Consequently, confidence intervals derived for mean NBD-predicted composite counts from 10g faecal samples would not satisfactorily explain the variation in observed counts from 10 x 5g composite samples, hence the need to include the additional egg count data from farms B.1 to B.22 and inclusion of quantity of faeces (x_2) as an explanatory variable (equation 2).

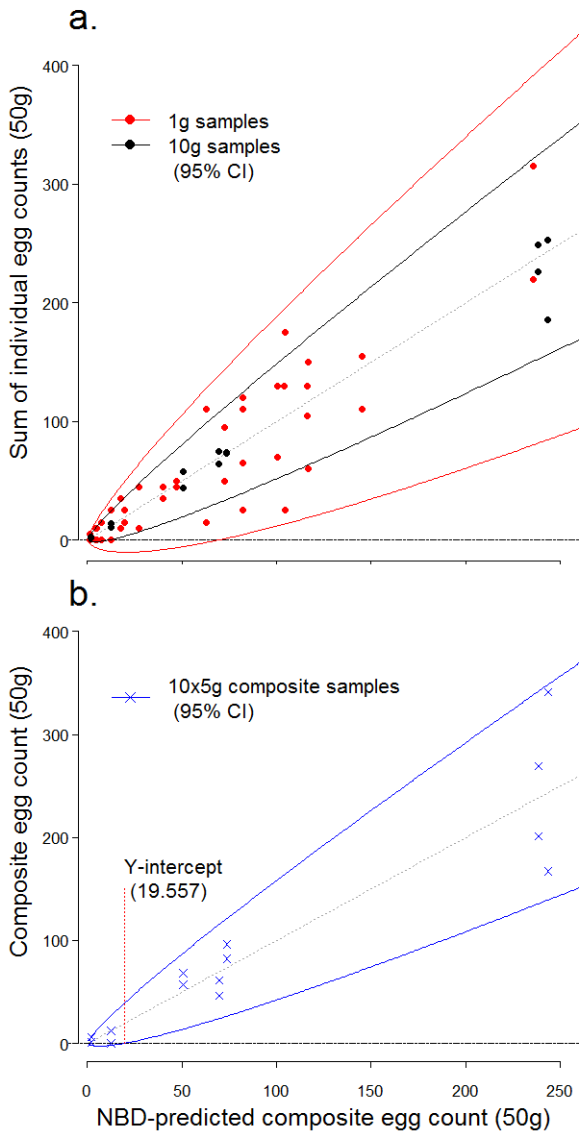

Figure 3.5: Comparison of model generated 95% confidence intervals to (a.) sum of observed individual egg counts (per 50g) based on individual counts for 1g and 10g faecal samples and (b.) observed composite egg counts for 10x5g composite samples.

Overdispersion, where the majority of parasitic burden within a population is present in a relatively small number of individuals, is a common feature of parasitic infections (Anderson and Gordon, 1982). The reasons for this are not fully understood, but have been linked to host-

susceptibility to infection, host-parasite interactions and likelihood of exposure to infection. The Negative Binomial Distribution (NBD) is frequently used to describe and model parasites within the host population (Anderson and May, 1991). Our results demonstrate that the NBD is an appropriate distribution for describing individual faecal egg count data for *F. hepatica* in cattle, as was also found to be the case previously in sheep (Daniel et al., 2012). There was a positive correlation between (log transformed) composite egg counts and group prevalence. A similar relationship has been shown previously between BMT antibody titres and herd prevalence as measured through both serum antibody ELISA and fluke egg counts (Salimi-Bejestani et al., 2005a). This suggests that as a screening tool, composite fluke egg counts can provide similar information to that of the BMT sample ELISA, since it both identifies infection at the herd/group level and gives an indication as to the level of prevalence. However, due to the wide range in confidence intervals of the fitted linear regression observed at low prevalence this should be interpreted with caution. As such, further investigation to better define this relationship would likely be of benefit, as it may ultimately allow composite egg counts to be used as part of a targeted threshold treatment or similar sustainable parasite control strategy.

3.6 Acknowledgements

We would like to thank the farmers we contacted for their time and willingness to be involved in this study and Ms. Katherine Allen for her technical expertise.

Chapter 4

Immune Responses in UK Dairy Heifers to Natural Challenge with *Fasciola hepatica*

4.1 Abstract

Fasciola hepatica is a parasitic trematode of global importance. Infection in livestock results in morbidity and significant economic losses, which in combination with widespread drug resistance and increasing disease prevalence mean new and sustainable methods of disease control are needed. Vaccination has been proposed as one such alternative, although trials in cattle to date have shown variable efficacy. Evidence from experimentally infected cattle indicates that *F. hepatica* modulates its host's immune response towards a non-protective type-2 response. Little, however, is known about the immune responses of cattle to naturally acquired infection.

To investigate this, cohorts of replacement dairy heifers (n=42) with no prior exposure to *F. hepatica* from three commercial dairy farms were sampled over the course of a grazing season. Exposure was determined through *F. hepatica*-specific serum antibody ELISA, with whole blood collected for haematological analysis and peripheral blood mononuclear cell (PBMC) isolation to examine leukocyte sub-populations and parasite specific proliferation and cytokine responses.

All calves on one farm acquired infection over the course of the grazing season, whilst cohorts on the remaining two farms remained negative. Data analysis by multivariable linear mixed effects modelling demonstrated a type-2 immune response was associated with infection, with increased interleukin(IL)-4 expression, IL-5 transcription and eosinophilia. Suppression of parasite specific PBMC proliferation was detected, suggesting parasite specific immune-modulation was present. Furthermore, increased IL-4 and decreased IFN-γ production by PBMCs in response to mitogen stimulation indicated a type-2 polarisation of the general immune response was present. These findings are consistent with those from experimental infections. Observed changes in the immune responses of naturally infected calves may also have wider implications for host immune responses to co-infection and disease control.

4.2 Introduction

Liver Fluke (*Fasciola hepatica*) is a parasitic trematode of major global importance. Infection with *F. hepatica* occurs in a wide range of vertebrate hosts (Boray, 1969), but is considered of greatest significance in livestock, particularly sheep and cattle. With over 600 million animals

thought to be infected globally, it is a major health and welfare issue and source of economic loss (Hillyer and Apt, 1997; Spithill et al., 1999).

Infection occurs through consumption of infective metacercariae present on herbage, such as grass. Once ingested, metacercariae pass to and excyst in the duodenum. These newly excysted juveniles (NEJs) then penetrate the intestinal wall and cross the peritoneum to reach the liver, penetrating the liver capsule and migrating through parenchymal tissue to gain access to the bile ducts where they mature and reside as adults (Andrews, 1999). Clinical presentation ranges from severe acute disease to chronic infection depending on the parasitic burden and stage of infection, although both result in severe morbidity and production losses (Boray, 1969; Sykes et al., 1980). Sub-clinical infections have also been shown to negatively affect weight gain, fertility and milk yield resulting in major economic losses (Charlier et al., 2007; Dargie, 1987; Howell et al., 2015).

In the UK, both incidence and prevalence of infection has increased over the last decade. This is largely attributed to climatic changes, particularly increases in ambient temperatures and rainfall, favouring the development of both *F. hepatica* and its intermediate snail host *Galba truncatula* and expanding disease range both temporally and spatially (Fox et al., 2011; van Dijk et al., 2010). In Europe, projected changes in weather patterns are predicted to support further increases in disease prevalence over the coming decades (Caminade et al., 2015).

Control of fasciolosis in livestock is currently based on the use of a limited number of anthelmintics, of which triclabendazole (TCBZ) is the most widely used due to its efficacy against both adult and migratory juvenile stages of the parasite (Boray et al., 1983; Smeal and Hall, 1983; Turner et al., 1984). There is increasing evidence of TCBZ resistance in *F. hepatica* populations both in sheep and cattle globally (Brockwell et al., 2014; Daniel et al., 2012; Gordon et al., 2012; Mitchell et al., 1998; Moll et al., 2000; Olaechea et al., 2011; Ortiz et al., 2013; Overend and Bowen, 1995; Thomas et al., 2000). The implications of drug resistance are well defined in livestock species, in terms of treatment, control and economic losses (Sargison and Scott, 2011; Sargison et al., 2010). There is therefore a need to develop novel approaches to control fasciolosis, with vaccination proposed as a potential adjunctive measure (Molina-Hernandez et al., 2015).

Current vaccine trials are focussed on inducing protective immunity through targeting specific proteins identified as essential for fluke survival within the host. A range of antigen candidates including cathepsin L proteases, glutathione S-transferase (GST), fatty acid binding proteins (FABP) and leucine aminopeptidase (LAP) have been trialled in a number of formulations and host species (cattle, sheep and goats) in both native and recombinant forms, with levels of protection reported ranging from 0-72% (Almeida et al., 2003; Toet et al., 2014). With such

huge variation in efficacy it is apparent that a greater understanding of the host immune response is required, specifically what constitutes a protective immune response.

The majority of work investigating the bovine immune response to *F. hepatica* has to date been done using experimental infections, either with a single dose challenge of metacercariae, or a trickle infection, where animals are challenged with multiple infectious doses administered over time to better simulate a natural challenge (Bossaert et al., 2000a). These studies have largely focussed on the presence or absence of type-1 (cell mediated) and type-2 (humoral) immune responses and how these change over the course of infection. In experimentally infected animals, the immune response is characterised by parasite specific IL-2 and IFN-γ production by *ex vivo* PBMCs between 1-3 weeks post infection in single dose and trickle infected animals, but are absent by 5 weeks post-infection (Clery and Mulcahy, 1998; Oldham and Williams, 1985). Similarly, parasite specific IL-2, IL-4 and IFN-γ production was observed in *ex vivo* mononuclear cells recovered from the hepatic lymph nodes of single dose infected cattle at 10-14 days post infection (Hoyle and Taylor, 2003). Increases in both parasite specific and mitogen stimulated PBMC proliferation is also observed in these early stages of infection, with peak responses at around 2 weeks post infection before returning to pre-infection levels as the disease progresses towards chronic infection (Bossaert et al., 2000b; Clery and Mulcahy, 1998; McCole et al., 1999; McCole et al., 1998a; Oldham and Williams, 1985). This progression towards a non-proliferative state in the chronic stages of infection is also associated with a polarisation towards a type-2 immune response with parasite specific antibody responses composed predominantly of the type-2 associated IgG1 isotype antibody and eosinophilia (Bossaert et al., 2000a; Bossaert et al., 2000b; Clery et al., 1996). Additionally, parasite specific $CD4^+$ T-cell clones isolated from chronically infected cattle (27 weeks post-infection onwards) demonstrated either a T_H0 or T_H2 phenotype as characterised by production of either IL-2, IL-4 and IFN-γ in combination, or IL-4 alone (Brown et al., 1994).

This immune response appears to have little to no protective effect; chronically infected cattle remain susceptible to re-infection, exhibiting impaired proliferative and cytokine responses when compared with previously uninfected animals responding to the parasite for the first time (Clery et al., 1996). Both IgG antibody titre and cumulative proliferative PBMC responses are shown to be positively correlated with parasite burden at post mortem, indicating that whilst these responses are features of the immune response, neither is associated with a protective effect (Clery et al., 1996; McCole et al., 1999; Salimi-Bejestani et al., 2008). Parasite longevity provides further evidence of the non-protective nature of the bovine immune response; cattle have been shown to remain infected following a single infectious challenge for months to years (Mulcahy et al., 1999), and there is also epidemiological evidence suggesting prevalence of infection increases with age (Gonzalez-Lanza et al., 1989).

This lack of protective immunity has led to the theory that *F. hepatica* induces a polarised type-2 immune response to facilitate its survival within the host (Dalton et al., 2013). Flynn and Mulcahy (2008b) demonstrated that *ex vivo* PBMCs taken from single dose infected cattle displayed increased production of the regulatory cytokines IL-10 and TGF-β in response to stimulation with both concanavalin A (ConA) and whole fluke homogenate as infection progressed from the juvenile migratory stages to adult stages in the bile ducts (10-12 weeks post infection). This study also demonstrated that neutralisation of these cytokines *in vitro* resulted in a reversion to pro-inflammatory cytokine production; IL-10 preferentially suppressed IFN-γ and TGF-β was found to suppress both IL-4 and IFN-γ. Similarly, Mendes et al. (2013) showed increased IL-10 and IL-4 and decreased IFN-γ transcription in hepatic tissue taken at post-mortem from animals with naturally acquired chronic infection, with a negative correlation between IL-10 and IFN-γ.

Fasciola hepatica has been shown to induce these regulatory responses through the production of a number of key antigens present in both excretory/secretory (E/S) products and surface tegument (TegAg). Much of this work has been carried out in mice and rats, where interactions between a number of components of E/S products including cathepsin L1 (CL1), peroxiredoxin (Prx) and glutathione S-transferases have been shown to affect the function and behaviour of antigen presenting cells (APCs), namely macrophages and dendritic cells (DCs) (Cervi et al., 1999; Donnelly et al., 2005; Donnelly et al., 2010). This has been shown to affect the adaptive immune response, with Prx shown to induce alternative activation of mouse macrophages *in vitro,* which when co-cultured with naïve $CD4^+$ T-cells, caused increased IL-4, IL-5 and IL-13 production and a suppression of IFN-γ (Donnelly et al., 2008). Similarly, *in vivo* stimulation of mouse peritoneal macrophages with either E/S antigen or TegAg resulted in activation of M2 macrophages, which subsequently caused $CD4^+$ T-cells to increase IL-5 and reduce IL-4 and IFN-γ production when co-cultured *in vitro* (Adams et al., 2014). Altered functionality has also been observed in mouse DCs when stimulated with *F. hepatica* TegAg, displaying reduced TNF-α, IL-10, IL-12p70 and nitrite production *in vitro*. Following re-inoculation of these TegAg-stimulated DCs into naïve mice, suppression of IFN-γ production by $CD3^+$ T-cells was observed when stimulated with anti-CD3 mAb *in vivo* (Hamilton et al., 2009). In cattle, parasite induced activation of macrophages to an M2 phenotype has also been demonstrated, with increased arginase activity, reduced nitric oxide (NO) production and increased IL-10 production in $CD14^+$ blood monocyte derived macrophages when taken from experimentally infected animals and stimulated with E/S antigen *in vitro* (Flynn and Mulcahy, 2008a). Both E/S antigen and TegAg have been shown to induce alternative activation in blood monocyte derived macrophages isolated from uninfected sheep *in vitro* (Flynn et al., 2007a; Hacariz et al., 2011). In addition to altered antigen presentation, M2 macrophages typically

exhibit reduced phagocytic and oxidative activity and increased Arginase-I (Arg1) enzyme activity, and are involved in fibrosis and tissue re-modelling. Combined with production of IL-10, this makes M2 macrophages potentially important in the regulation of local inflammatory responses.

Where there is evidence of a protective immunity, cellular responses appear to be important. In experimentally infected cattle, a negative correlation between fluke length and PHA-induced eosinophil recruitment has been demonstrated (Bossaert et al., 2000b), whilst major basic protein purified from bovine eosinophils has been shown to kill NEJs *in vitro* (Duffus and Franks, 1980). In rats, evidence from the early intestinal and peritoneal stages of infection suggest a local immune response comprised of macrophage and eosinophil mediated antibody-dependent cell mediated-cytotoxicity (ADCC) is protective (Van Milligen et al., 1999), whilst IgG1 and IgG2 isotype antibodies have also been shown to be an important feature of this protective mechanism (Van Milligen et al., 1998). In cattle, IgG2 is known to be induced by type-1 responses, specifically IFN-γ (Estes et al., 1994) and is an important isotype in facilitation of cytotoxic action by bovine macrophages through IgG2-specific Fc receptors (Tizard, 2013). Where a protective immune response is induced by vaccination in cattle, fluke burden has been shown to be negatively correlated with IgG2 isotype antibody titre and avidity (Mulcahy et al., 1998). Since protective immune responses in vaccinated animals are also associated with reduced arginase activity in macrophages compared to unvaccinated control animals (Golden et al., 2010), this would suggest that vaccine-induced protective immunity is associated with ADCC responses, although type-2 responses could still have a role in protection.

One additional and important feature of *F. hepatica* infection in the wider context of herd health and disease control is its ability to modulate the immune system generally resulting in so called bystander effects on the host's ability to respond to co-infecting pathogens. In mice co-infected with *F. hepatica* and *Bordetella pertussis,* a reduction in vaccine efficacy against the latter was shown to be associated with a corresponding reduction in *B. pertussis* specific IFN-γ and delayed clearance of bacteria from lung tissue (Brady et al., 1999). In cattle co-infected with *F. hepatica* and *Mycobacterium bovis,* parasite-induced alternative macrophage activation has been associated with a reduction in IFN-γ production in whole blood cultures stimulated with PPD and ESAT-6 (Garza-Cuartero et al., 2016). Furthermore, these alternatively activated macrophages have been shown to exhibit reduced mycobacterial uptake, with a subsequent reduction in the overall mycobacterial burden in co-infected animals. This effect has also been shown to reduce the sensitivity of the single intradermal comparative cervical tuberculin (SICCT) used to diagnose *M. bovis* in co-infected animals, potentially resulting in an under-diagnosis of this pathogen in the UK (Claridge et al., 2012; Flynn et al., 2009). In cattle, *F.*

hepatica infection has also been linked to carriage of *Salmonella dublin* (Aitken et al., 1979; Vaessen et al., 1998).

Although there is already considerable information about the bovine immune response to *F. hepatica* infection, the majority of this comes from experimental infection studies. Little is known of the responses to naturally acquired infection, specifically, the extent of parasite-induced polarisation and/or immune-modulation making it unclear how vaccines should be used in the field. Improving our knowledge in this area will allow us to better understand how vaccines should be implemented in terms of the timing of administration, and whether they can be given to animals that have been exposed to natural infection. This would also help improve our understanding of the immunological and epidemiological impact of *F. hepatica* on co-infecting pathogens.

The aim of this study was to define the immune responses of dairy heifers to primary challenge with *F. hepatica*, acquired naturally over the course of their first grazing season.

4.3 Materials and Methods

Three commercial dairy farms located in Cheshire and north Wales were recruited to this study. These herds were identified as *F. hepatica* positive through bulk milk tank (BMT) antibody ELISA and composite faecal counts in the adult cattle on the farms (Salimi-Bejestani et al., 2005a). On each farm, cohorts of replacement dairy heifer calves were recruited (n=42; 17, 17 and 8 animals from farms A, B and C respectively) aged 90-377 days (mean=218.5, SD ±62.0). These animals had no prior exposure to *F. hepatica*, which was confirmed through serum antibody ELISA (Salimi-Bejestani et al., 2005b), and composite fluke egg counts prior to turn-out. Animals were screened for bovine viral diarrhoea (BVD) antigen to check for persistently infected animals ahead of study commencement as this could have affected our results by altering immune responses (Radostits and Littlejohns, 1988).

Animals were sampled on a monthly basis over the course of their first grazing season from turn-out in spring (April-May) through to housing in autumn (October-November) 2013. On each occasion blood was collected via jugular venepuncture into both plain and EDTA coated vaccutainers. Faecal samples were collected rectally and animal weights estimated using a commercial Holstein weight tape (Nasco, Fort Atkinson USA) based on heart girth measurements (Heinrichs and Lammers, 1998). Additionally, BMT samples were taken at each visit for analysis of *F. hepatica* infection within the milking herd. Animals were kept under normal farm management conditions at each premises, including administration of vaccines and treatments typical of each farm's disease control programme. For Farm A this included three consecutive treatments of the cohort group with a pour-on ivermectin preparation at the 2nd, 5th and 7th months of sampling, whilst cohorts from Farms B and C each only received a

single treatment with ivermectin at the 5th and 6th months of sampling, respectively (APPENDIX D).

4.3.1 Fluke Egg Counts

Faecal samples were stored at 4°C prior to parasitological analysis as described previously (Section 2.7). Samples were examined for evidence of *F. hepatica* eggs prior to turnout through composite sample sedimentation, then individually from the point of sero-conversion onwards using a standard sedimentation technique on 10g of faeces (Anon., 1986). All animals were individually assessed at the final time point for evidence of *F. hepatica* eggs irrespective of sero-conversion status. In addition to fluke egg sedimentations, nematode infections were assessed using a standard McMaster's technique for all animals at all time points (Thienpont et al., 1986).

4.3.2 *F. hepatica* Serum and BMT Antibody ELISAs

Clotted blood tubes were centrifuged at 2,000g for 5 min to separate the serum fraction for storage at 4°C ahead of analysis. Infection status was determined at each time point by measuring *F. hepatica* specific serum IgG antibody via ELISA as described previously (Salimi-Bejestani et al., 2005b)(Section 2.6), with values expressed as percent positivity (PP) of known positive control sample. For this study, a positive cut-off value of 20PP was used, with a diagnostic sensitivity of 95% and specificity of 99% (Salimi-Bejestani et al., 2005b). For BMT samples a previously defined positive cut-off value of ≥27P was used, giving a diagnostic sensitivity of 96% and specificity of 80% (Salimi-Bejestani et al., 2005a).

4.3.3 Preparation of *F. hepatica* Antigens

Adult *F. hepatica* tegument and somatic antigen fractions were prepared using previously described methods (Hamilton et al., 2009; Hillyer, 1980; Oldham and Williams, 1985); briefly, freshly acquired adult fluke collected from infected liver were incubated overnight to purge caecal contents, fluke were washed three times in D-PBS (Sigma-Aldrich, St. Louis USA. Tegument antigen (TegAg) was prepared by placing fluke in a solution of D-PBS with 1% Nonidet P-40 (BDH Chemicals, Poole UK), 1ml per fluke, and gently agitated for 1 hour at 4°C. Pierce® Detergent Removal spin columns (Thermo Fisher Scientific, Waltham USA) were used to remove Nonidet P-40 detergent from the resulting TegAg containing solution following manufacturer's specifications (Thermo Fisher Scientific, Waltham USA) (Section 2.5.2).

Preparation of somatic antigen (SomAg) was performed following tegument depletion (Section 2.5.3). Fluke were washed in ice cold D-PBS and snap frozen overnight at -80°C, then homogenised and diluted in D-PBS, 0.5ml per fluke, and left to stand overnight at 4°C. The

resulting supernatant was removed, centrifuged at 12,000g for 30 min at 4^{o}C and filter sterilised. Both antigen fractions were then assessed for protein concentration and presence of endotoxin prior to storage at -80^{o}C (Sections 2.5.4 & 2.5.5).

4.3.4 Total Leukocyte Counts and Haematology

EDTA treated whole blood was used for leukocyte enumeration (Section 2.11.1). Total leukocyte counts were performed on whole blood diluted 1:20 in a 2% acetic acid and 1% gentian violet using a 102 FastRead^{TM} multi chamber haemocytometer (immune systems, Paignton UK) to give total leukocyte counts per ml peripheral blood.

Thin blood smears were then prepared using Wright's stain as per manufacturer's instructions (Procedure No. WS, Sigma-Aldrich, St. Louis USA) and examined under oil immersion at x100 magnification. The resulting differential counts for eosinophils (Eϕ), neutrophils (Nϕ), lymphocytes (Lϕ), monocytes/macrophages (Mϕ) and basophils (Bϕ) were used to calculate estimated counts for each phenotype per ml of peripheral blood as a proportion of total leukocyte counts.

4.3.5 PBMC Purification

PBMCs were isolated from whole blood in a lateral flow hood under sterile conditions using Optiprep^{TM} (Sigma-Aldrich, St. Louis USA) following manufacturer's recommendations (Section 2.8). Optiprep^{TM} was added to, and mixed with, EDTA treated whole blood, 1.3ml per 10ml of blood, in a 50ml falcon tube onto which 1ml 20mM tricine-buffered saline was layered. Samples were centrifuged at 1,000g for 35 min at 20^{o}C with the brake off, following which the middle aqueous layer containing PBMCs was harvested and washed by adding PBS with 0.1% EDTA (Lonza, Bazel Switzerland) to a maximum volume of 20ml. Samples were centrifuged at 350g for 8 min at 20^{o}C. Resulting supernatants were discarded and cell pellets re-suspended in 2ml of 0.9% NH_4Cl and gently agitated for 1 min at room temperature to lyse residual erythrocytes. Samples were then washed in 20ml PBS EDTA and centrifuged at 150g for 8 min at 20^{o}C twice, with purified PBMCs then re-suspended in 5ml cell culture medium consisting of RPMI supplemented with 10% heat inactivated foetal calf serum (Sigma-Aldrich, St. Louis USA) and 100µg/ml penicillin and streptomycin (Sigma-Aldrich, St. Louis USA). Sample concentration and cell viability was assessed using a 102 FastRead^{TM} multi chamber haemocytometer following a 1:50 sample dilution in PBS EDTA and Trypan blue solution.

4.3.6 Flow Cytometry

One colour indirect immunofluorescence was performed on PBMCs as previously described (Rosbottom et al., 2007)(Section 2.11.2). PBMCs were adjusted to a concentration of 1 x 10^{7}

cells/ml in PBS EDTA and pipetted out into 96-well round bottomed mixing plates (5 x 10^5 cells per well) and centrifuged at 500g for 5 min. Supernatant was discarded and cells incubated with 25µl of primary mouse anti-bovine cell surface receptor monoclonal antibodies diluted in PBS with 1% BSA, 0.1% sodium azide at concentrations shown in table 4.1. Plates were incubated on ice for 10 min after which cells were washed twice with PBS/BSA/azide. FITC-conjugated goat anti-mouse IgG monoclonal antibody (Sigma-Aldrich, St. Louis USA) was then added at a 1:200 dilution in PBS/BSA/azide , 25µl per well, and incubated in the dark on ice for 10 min, washed, and fixed in 1% neutral buffered formalin ahead of storage in 200µl of PBS with 0.1%BSA/sodium azide at 4^{o}C overnight.

Samples were analysed using a MACSQuant® analyser (Miltenyi Biotech Ltd.) by fluorescence emission at 525/50nm following excitation at 488nm. PBMCs were selected through gating of forward and side scatter values, and percentage of each cell phenotype was measured by gating for FITC fluorescence. Resulting percentage values were used to calculate estimate counts per ml of peripheral blood as a proportion of lymphocytes counts as determined previously (Section 4.3.4).

4.3.7 PBMC Cultures

Purified PBMCs were incubated with either the mitogen ConA (5µg/ml), *F. hepatica* SomAg or TegAg (both 25µg/ml) or as unstimulated medium controls to assess proliferative and cytokine responses (Williams et al., 2000). PBMC samples were adjusted to a concentration of 2 x 10^6 per ml in RPMI with 10% FCS and 100µg/ml penicillin and streptomycin.

For proliferation assays (Section 2.8.1), cells were cultured in triplicate in 96-well U-bottomed plates (Corning Life Sciences, Corning USA) for 5 days at 37^{o}C, 5% CO_2, 2 x 10^5 cells per well. After 5 days, cultures were pulsed with 1µCi of [H^3] tritiated thymidine (Perkin Elmer, Boston USA) for 5 hours then harvested onto glass filter mats (Perkin Elmer, Boston USA) and embedded in scintillation wax (Perkin Elmer, Boston USA). β-particle emission counts were measured using a MicroBeta^2 plate counter (Perkin Elmer, Boston USA). The stimulation index (SI) was calculated as the fold increase in β-particle decay compared to medium controls. Where the SI for the ConA positive control was <2, proliferation values for both mitogen and antigen stimulated cultures were excluded.

Cytokine production and transcription was assessed in animals which sero-converted (Farm A) (Section 2.9). For this, PBMCs were incubated with ConA, SomAg or medium alone for 48 hours at 37^{o}C, 5% CO_2 in 24 well cell culture plates (VWR, Radnor USA) after which both PBMCs and culture supernatants were harvested for qPCR and cytokine expression analysis respectively. PBMCs were stored in RNAlater (Sigma-Aldrich, St. Louis USA) along with culture supernatants at -20^{o}C.

Table 4.1: Primary monoclonal antibody dilutions for indirect immunofluorescence staining of PBMCs. Dilution indicates the relative volumes for antibody stock and dilution buffer (PBS with 1% BSA and 0.1% sodium azide)

Cell type	Antibody	Dilution
$CD4^+$	IL-A11	1:10
$CD8^+$	(Serotec MCA837GA) CC-63	1:100
$WC1^+$	CC-15	1:10
$CD14^+$ monocytes	CCG-33	1:10

4.3.8 Cytokine Production and Transcription

Cytokine expression was assessed by measuring either protein concentration in culture supernatants or mRNA transcription in PBMCs. Interferon(IFN)-γ and interleukin(IL)-4 were measured in supernatants using commercially available ELISAs following manufacturer's protocols (MCA5638KZZ & MCA5892KZZ respectively, AbD Serotec, Raleigh USA)(Section 2.9.1 & 2.9.2.1). Interleukin(IL)-10 production was measured in medium controls and SomAg stimulated cells using a previously validated sandwich ELISA (Kwong et al., 2002)(Section 2.9.3), and transforming growth factor-β (TGF-β) was measured in medium controls and SomAg stimulated wells using a commercially available ELISA shown to detect bovine TGF β (Abbott et al., 2005). Samples were acid treated to convert TGF- β to its bio-active form following manufacturer's recommendations (TB196 Technical Bulletin, Promega, Madison USA) (Section 2.9.4). Paired samples were used to investigate difference in TGF-β production in early versus late stage infection; samples for each individual animal were selected at, or close to sero-conversion to represent early stage infection, and from the last available time point to represent chronic stages of infection.

Cultured PBMCs were assessed for Interleukin-2 (IL-2) and interleukin-5 (IL-5) gene transcription through quantitative real-time (q)PCR analysis (Section 2.10). RNA extraction was performed using the RNeasy Mini kit following manufacturer's specifications (QIAGEN, Limburg Netherlands), following which mRNA concentration was quantified with RiboGreen® (Invitrogen Life Technologies, Grand Island USA). Genomic DNA digestion and cDNA template synthesis was then performed on 1µg of mRNA template for each sample using Quantitect Reverse transcriptase kit (QIAGEN, Limburg Netherlands) and stored at -20^{o}C ahead of qPCR analysis. Primer sequences for 28s, IL-2 and IL-5 were taken from previous studies (Freeman et al., 2008; Rosbottom et al., 2008; Rosbottom et al., 2007) and used under conditions described in table 4.2. qPCR analysis was performed using a standardised protocol with SYBR Green (Bioline reagents Ltd., London, UK) as described previously (Hansen et al., 2011)(Section 2.10.3). Briefly, master mix was prepared at a volume of 19µl per reaction comprising 10µl 2× SensiMix with SYBR Green I, with primers diluted in Ambion® nuclease-free water (Thermo Fisher Scientific, Waltham USA) to the specified concentration (table 4.2). To this, 1µl of

sample cDNA template was added at the specified dilution in nuclease free water (table 4.2). Samples were run in duplicate alongside cDNA standards diluted in 100ng/µl yeast tRNA (Invitrogen Life Technologies, Grand Island USA) to prevent aggregation. Reactions were performed using a DNA Engine opticon 2 continuous Fluorescence detector: Following an initial denaturation step occurred at 95°C for 10 min, samples were submitted to 35-40 cycles (table 4.2) of 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 15 seconds, with fluorescence measured after each cycle.

Upon completion of PCR, a melting curve analysis was performed from 50-95°C to confirm the specificity of the amplification products. Sample copy numbers were determined by linear regression of standard concentrations following adjustment of Ct cut-off values to the log-linear phase of amplification. For 28s, where values for two concentrations differed, the 1:1000 sample dilution was preferred over the 1:100 dilution (table 4.2) due to the lower risk of reaction inhibition. Results for IL-2 and IL-5 transcription were then given as relative expression against 28s housekeeper gene (per million copies).

Table 4.2: qPCR reaction details for bovine cDNA sequences. Sample dilution indicates the relative volumes of cDNA stock samples and nuclease free water. Analysis of 28s was carried out at both quoted concentrations. Cycle number refers specifically to the PCR reaction (Section 4.3.8)

Target gene	Primer concentration (µM)	NCBI accession No.	Sample dilution	Cycle number
28s	0.2	AF154866	1:100; 1:1000	35
IL-2	0.6	M12791	1:10	40
IL-5	0.2	EU915048.1	1:10	35

4.3.9 Statistical Analysis

Data analysis was performed using linear mixed effects models in R. Response variables (Y) were chosen to determine immune responses associated with infection/exposure to *F. hepatica*; antibody PP value (Ab) was used to assess responses over the course of infection due to their continual increase over time (figure 4.1a), whilst the change in antibody PP value (ΔAb) between time points was used to indicate early stages of infection, since the greatest changes in antibody levels were detected at or soon after sero-conversion (figure 4.2). When assessing immune responses against ΔAb, data was sub-setted based on sero-conversion status, with positive and negative values considered separately (lme_3, 7 & 8; table 4.3). Models considering only sero-negative measurements (lme_6, 7 & 8) served to investigate potential physiological and/or age related changes. Insufficient data was available to model sero-negative measurements for animals from farm A in isolation.

In addition to Ab and ΔAb, PBMC proliferation responses to SomAg and TegAg were modelled as response variables to identify associated immune responses (lme_4 & 5; table 4.3). Immunological parameters, farm location, animal age, weight and days exposure (days at

pasture) were all modelled as fixed effects, whilst individual animal identity was modelled as a random effect to account for inherent variation in responsiveness between different individuals. Prior to model fit and refinement, Box-Cox transformations were performed on response variables using the lambda value (λ) obtained from evaluation of the fixed effects component of the model (Faraway, 2005). For models where Ab was used as the response variable, an "AR-1 autoregressive correlation matrix" was fitted to account for the increased relatedness of measurements resulting from repeated sampling, whilst missing (NA) values were considered to be "dropouts completely at random" (DCAR) (Everitt and Hothorn, 2010). Model refinement was carried out using a stepwise Akaike information criterion (AIC) selection method using complete case data with maximum likelihoods (ML). The refined model was then fitted to all available data using restricted maximum likelihoods (REML) to give a final model analysis which was checked for goodness of fit and normality using residual and qq-plots, respectively. Details of the structure of each statistical model (numerically referred to as "lme_x") are shown in table 4.3.

To assess TGF-β expression in early versus late stage infection results were analysed using paired two-tailed T-tests, whilst measurements from different culture conditions for each time point were analysed using unpaired two-tailed T-tests.

All procedures and experiments for this study were approved by the University of Liverpool ethics committee and adhered to the conditions of the project license granted by the UK Home Office. All farm data was stored in accordance with the Data Protection Act (1998).

4.4 Results

4.4.1 *F. hepatica* Infection and Observed Immune Responses

All animals remained healthy throughout the study, with no clinical signs observed as a result of *F. hepatica* infection, nematode burden or other health related condition. All animals tested negative for BVD antigen at the start of the study. Monthly BMT samples taken from all three farms yielded positive *F. hepatica* antibody ELISA results for the duration of the study (*APPENDIX D*).

Sero-conversion was observed in all 17 animals on farm A. PP values increased over the course of the study, and reached 37-98PP at the final time point (figure 4.1a). Whilst PP value (Ab) increased over the course of the study, greatest changes in antibody PP value (ΔAb) were seen at or soon after sero-conversion, with smaller changes in PP value seen later in infection (figure 4.2). Fluke eggs were detected in 10 of the 17 animals from farm A by the final time point, with counts in all cases less than 1 egg per gram of faeces. No correlation was found between fluke egg counts and ELISA PP value (*APPENDIX D*). Paramphistome eggs were also

Table 4.3: Linear mixed effects model structures. Response variable (Y) indicates the subject of each respective model. Box-Cox value (λ) is the optimal transformation factor for the response variable to ensure linear fit to the proposed fixed-effect variables. Where *nil* is quoted, transformation was not required. The abbreviation "Leuks" refers to Leukocytes per ml of peripheral blood, "prolifn" to PBMC proliferation, whilst "prdn" and "trxn" refer to cytokine production and transcription, respectively. Additionally, for cytokines, square parentheses [] indicate antigen used for *ex vivo* stimulation.

Linear mixed effect model	Response variable (Y)	Box-Cox value (λ)	Data analysed	Initial fixed-effect explanatory variables (x)	Final explanatory variables (x) following stepwise AIC selection
lme_1	Ab	-0.2	All data	Farm + Days exposure + Age + Weight + Leuks: (Eϕ + Nϕ + CD4 + CD8 + WC1 + CD14) + CD4:CD8 ratio + prolifn: (ConA + SomAg + TegAg)	Farm + Days exposure + Leuks: (Eϕ + CD4 + CD8 + WC1) + prolifn: (SomAg + TegAg)
lme_2	Ab	0.2	Farm A	Days Exposure + Age + Weight + Leuks: (Eϕ + Nϕ + CD4 + CD8 + WC1 + CD14) + CD4:CD8 ratio + prolifn: (ConA + SomAg + TegAg) + prdn: (IFNγ [ConA] + IFNγ [SomAg] + IL-4 [ConA] + IL-4 [SomAg] + IL-10 [Med] + IL-10 [SomAg]) trxn: (IL-2 [ConA] + IL-2 [SomAg] + IL-5 [ConA] + IL-5 [SomAg])	Days Exposure + Weight + Leuks: (Eϕ) + SomAg prolifn + PBMC cytokine prdn: (IFNγ [ConA] + IFNγ [SomAg] + IL-4 [ConA] + IL-10 [Med] + IL-10 [SomAg]) + PBMC cytokine trxn: (IL-2 [ConA] + IL-5 [SomAg])
lme_3	ΔAb	*nil*	Sero-positive values; Farm A	*(see lme_2)*	Days exposure + Leuks: (CD4 + WC1) + prolifn: (SomAg + TegAg) +IL-4 [ConA] prdn + IL-5 {SomAg] trxn
lme_4	SomAg	0.4	Farm A	Days exposure + Age + Weight + Ab + ΔAb + Leuks: (Eϕ + Nϕ + CD4 + CD8 + WC1 + CD14) + CD4:CD8 ratio + prolifn: (ConA + TegAg) + prdn: (IFNγ [ConA] + IFNγ [SomAg] + IL-4 [ConA] + IL-4 [SomAg] + IL-10 [Med] + IL-10 [SomAg]) trxn: (IL-2 [ConA] + IL-2 [SomAg] + IL-5 [ConA] + IL-5 [SomAg])	Days exposure + ΔAb + Leuks: (CD4 + CD8) + CD4:CD8 + prolifn: (ConA + TegAg) + prdn:(IFNγ[SomAg] + IL-4[ConA])
lme_5	TegAg	0.3	Farm A	Days exposure + Age + Weight + Ab + ΔAb + Leuks: (Eϕ + Nϕ + CD4 + CD8 + WC1 + CD14) + CD4:CD8 ratio + prolifn: (ConA + SomAg) + prdn: (IFNγ [ConA] + IFNγ [SomAg] + IL-4 [ConA] + IL-4 [SomAg] + IL-10 [Med] + IL-10 [SomAg]) trxn: (IL-2 [ConA] + IL-2 [SomAg] + IL-5 [ConA] + IL-5 [SomAg])	Ab + Leuks: (CD4 + CD8 + CD14) + CD4:CD8 + prolifn: (ConA + SomAg) + IL-10[SomAg] prdn + trxn: (IL-2[ConA] + IL-5[ConA] + IL-5[SomAg])
lme_6	Ab	-0.2	Farms B & C	*(see lme_1)*	Farm + Days exposure + SomAg prolifn + Leuks: (Eϕ + CD4 + CD8 + WC1)
lme_7	ΔAb	*nil*	Sero-negative values; Farms A, B & C	*(see lme_1)*	Farm + Leuks: (CD4 + CD8) + CD4:CD8 + prolifn: (SomAg + TegAg)
lme_8	ΔAb	*nil*	Farms B & C	*(see lme_1)*	Leuks: (Eϕ + CD8)

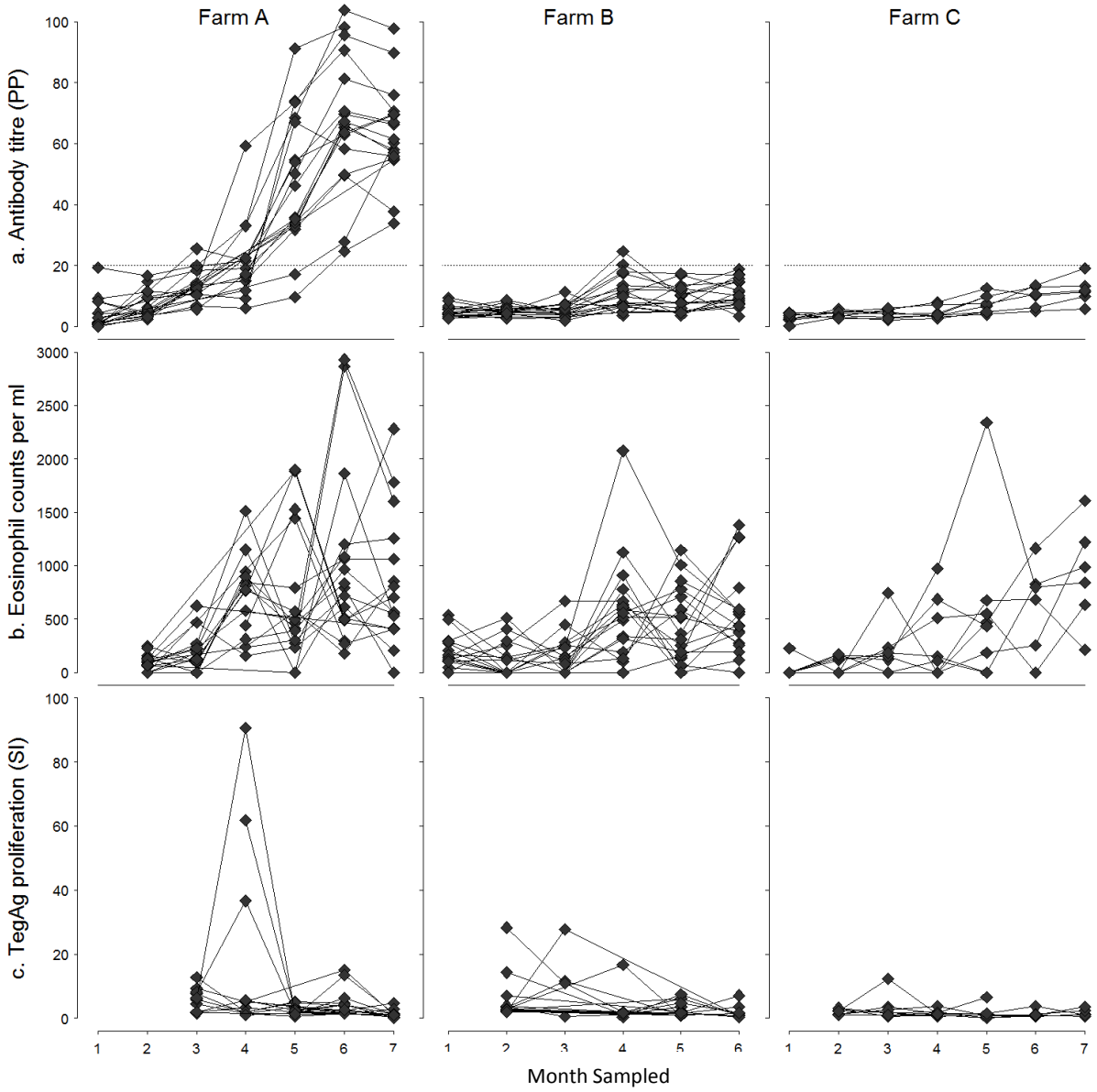

Figure 4.1: Immune parameters of individual animals over study period. (a.) Antibody PP values with positive cut-off value (PP=20) denoted by horizontal line (b.) Eosinophil counts in peripheral blood and (c.) Tegument antigen specific PBMC proliferation. Sample number denotes the time point for sequential sampling on each farm.

observed in low numbers in seven of these animals at the final time point, six animals were positive for both *F. hepatica* and Paramphistome eggs.

Two animals from farm B had PP values above the 20PP cut-off value at single time points. Fluke egg counts remained negative throughout the study on farms B and C.

Low numbers of nematode eggs were detected in faecal samples on all three farms, with only two positive samples detected on Farm A at a single time point. Both trichostrongyle and *Nematodirus* spp. eggs were found in samples from calves from farm B, with *Nematodirus* spp. eggs only observed towards the end of the grazing season (*APPENDIX D*).

Eosinophil counts increased in animals on all three farms over the course of the study (figure 4.1b). On farm A, the PBMC proliferation responses to SomAg were similar to those obtained

with TegAg stimulation (figure 4.1c). These were variable both between and within animals at different time points, with greatest proliferative responses observed in animals from Farm A. Similar variation was also observed for cytokine responses in *ex vivo* PBMC cultures both in response to ConA and SomAg stimulation (figure 4.3). ConA-stimulated IFN-γ production was observed following sero-conversion, with evidence of ConA stimulated IL-4 production and IL-5 transcription also. In response to stimulation with SomAg, IFN-γ production was largely absent, whilst low levels of both IL-4 production and IL-5 transcription were observed in the month prior to sero-conversion.

No significant difference was found for IL-10 production over the course of infection, or between levels of TGF-β expression in early versus late stage infection in either medium control or SomAg stimulated PBMC cultures (p=0.791 & 0.828, respectively) (figure 4.4), nor was any significant difference found between TGF-β levels in medium control or SomAg stimulated cultures at either time point (p=0.291 & 0.306, respectively).

4.4.2 Model Outputs

Signalment (i.e. age and weight) were not found to be correlated with any response variables modelled, and were subsequently dropped from most analyses during stepwise AIC selection.

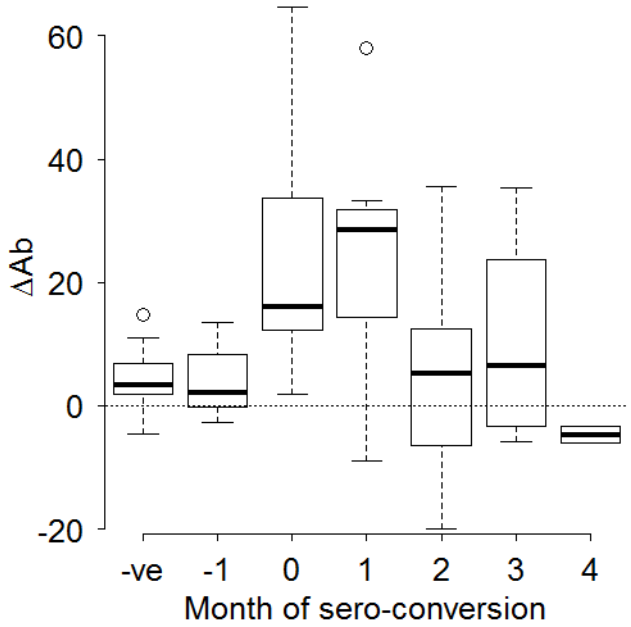

Figure 4.2: Change in antibody PP value (ΔAb) over the course of infection for farm A. Positive values indicate an increase in Ab PP value from one time point to the next, whilst negative values denote a decrease in Ab PP value compared to the previous month. 1 month prior to sero-conversion (-1) is considered separately from other sero-negative values (-ve); infection may have occurred at this time point, since positive Ab PP-values are first observed from 2-4 weeks post-infection (Salimi-Bejestani et al., 2005b).

4.4.2.1 Antibody PP value (Ab)

Statistically significant, positive coefficient values (β) were obtained between antibody PP value (Ab) and days exposure in models using all available data and data from farm A only, but not for farms B and C (lme_1, 2 & 6; table 4.4). In lme_1, statistically significant negative coefficients for both farms B and C were also observed.

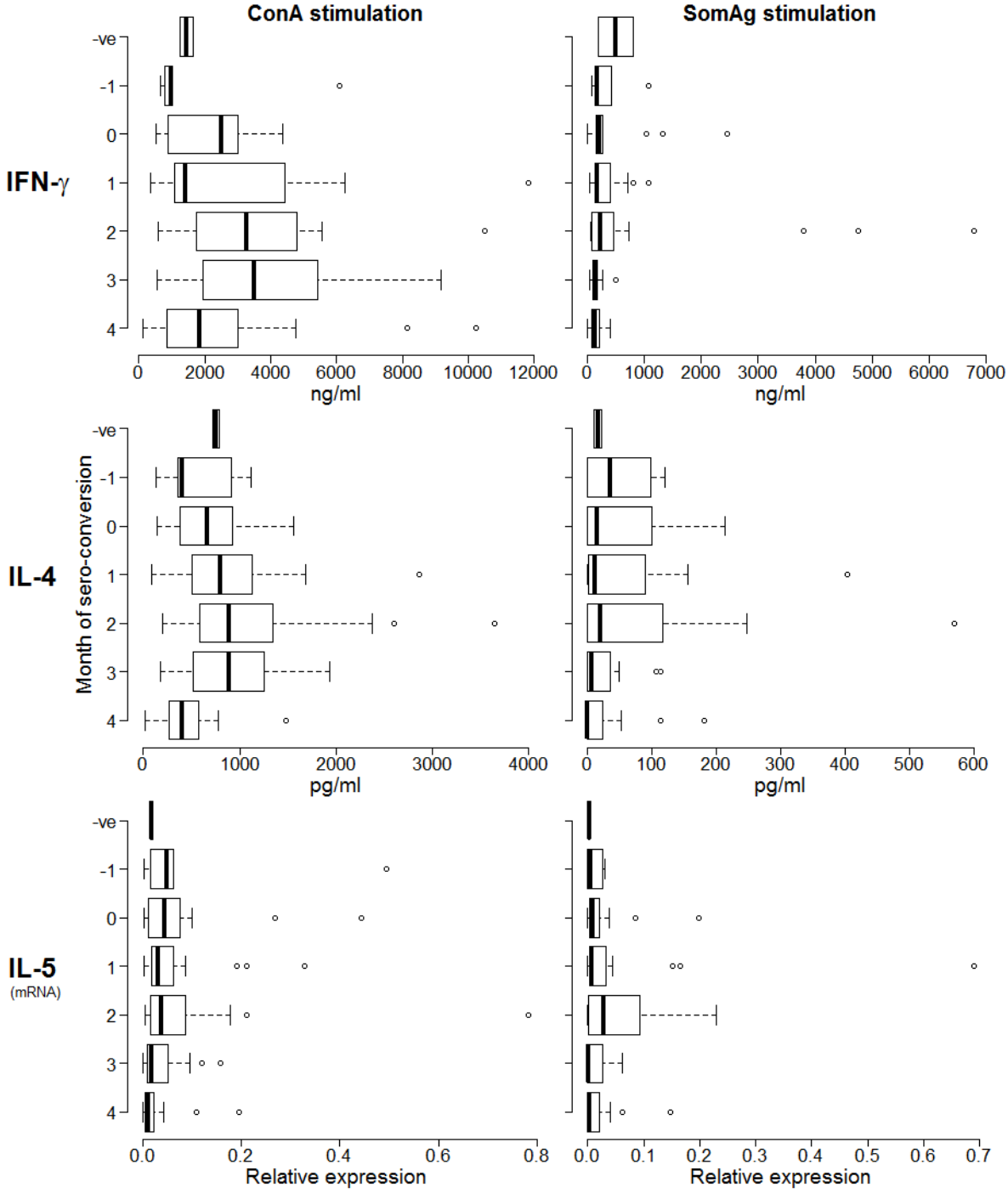

Figure 4.3: *Ex vivo* PBMC culture cytokine profiles for Farm A (1). Cytokine production (IFNγ and IL-4) or transcription (IL-5) by *ex vivo* PBMCs in response to stimulation with either ConA (left) or SomAg (right). Boxplots represent the cytokine production/expression at different months of sero-conversion. Outputs for 1 month prior to sero-conversion (-1) are shown separately from other sero-negative values (-ve) since infection could be present at this time point.

Statistically significant positive coefficient values were found between Ab PP values and eosinophil counts when using all available data (lme_1). A positive coefficient value was obtained between $CD8^+$ T-cell counts for all data (lme_1), and for farms B and C (lme_6). A negative coefficient value was returned for $WC1^+$ γδT-cell counts when using all data (lme_1). SomAg specific proliferation had negative coefficients in relation to Ab in all instances (lme_1, 2 & 6). With respect to cytokine production and transcription, when using data from farm A only, positive coefficients were returned for both ConA stimulated IL-4 production and SomAg stimulated IL-5 transcription, and there was a negative coefficient value for ConA stimulated IFN-γ production (lme_ 2).

4.4.2.2 Change in Antibody PP value (ΔAb)

Where change in antibody PP-value (ΔAb) was modelled as the response variable (Y), a statistically significant negative coefficient value was found for days exposure, whilst positive coefficient values were returned for ConA stimulated IL-4 production and SomAg stimulated IL-5 transcription in antibody positive animals (lme_3).

A positive coefficient value was found for TegAg stimulation and a negative coefficient value for SomAg for sero-positive values (lme_3). These relationships between Ab and parasite specific proliferation were also observed when using sero-negative data (lme_7).

In sero-negative samples, a positive coefficient value was also found between ΔAb and $CD8^+$ T-cells (lme_7). Although stepwise AIC selection produced a valid model when using sero-negative data from farms B & C, no explanatory variable had a significant relationship with ΔAb (lme_8).

4.4.2.3 Parasite-specific PBMC Proliferation

When SomAg specific proliferation was modelled as the response variable (lme_4), a statistically significant negative coefficient value was given for days exposure, whilst positive coefficient values were found for both ConA specific IL-4 production and circulating $CD4^+$ T-cells, with a negative coefficient found for $CD8^+$ T-cells.

When TegAg was modelled as the response variable (lme_5), a statistically significant, negative coefficient value was found for $CD8^+$ T-cell counts and a positive coefficient value for $CD14^+$ peripheral blood monocytes.

In both cases, there was a correlation between SomAg, TegAg and ConA stimulated proliferation (lme_4 & 5).

Table 4.4: Linear mixed effects model outputs. Statistically significant explanatory variables derived from linear mixed effects modelling (table 4.3) with Coefficient (β), Standard error (SE) and P-values.

Model and response variable (Y) *Data used*	Explanatory variable (x)	Coefficient value (β)	Standard error (SE)	P-value
lme_1 (Ab) *All data*	Farm B	-0.687	0.0765	0.0000
	Farm C	-0.874	0.0867	0.0000
	Days exposure	0.00520	0.000592	0.0000
	Eϕ/ml	0.0000964	0.0000420	0.0237
	CD8 /ml	0.000244	0.0000998	0.0163
	WC1/ml	-0.0000976	0.0000438	0.0278
	SomAg	-0.00618	0.00270	0.0237
lme_2 (Ab) *Farm A*	Days exposure	0.0270	0.00283	0.0000
	SomAg	-0.0116	0.00544	0.0395
	[ConA] IFNγ	-0.000129	0.0000559	0.0268
	[ConA] IL-4	0.000516	0.000242	0.0398
	[SomAg] IL-5	0.00304	0.00115	0.0122
lme_3 (ΔAb) *Farm A, post sero-conversion*	Days exposure	-0.354	0.107	0.0041
	SomAg	-2.794	1.211	0.0340
	TegAg	2.254	1.064	0.0493
	[ConA] IL-4	0.0118	0.0509	0.0338
	[SomAg] IL-5	0.0710	0.0293	0.0269
lme_4 (SomAg) *Farm A*	Days exposure	-0.0126	0.00400	0.0036
	CD4/ml	0.000987	0.000420	0.0256
	CD8/ml	-0.00345	0.00154	0.0332
	ConA	0.00320	0.00105	0.0046
	TegAg	0.0955	0.00963	0.0000
	[ConA] IL-4	0.00108	0.000326	0.0024
lme_5 (TegAg) *Farm A*	CD8/ml	-0.00428	0.00206	0.0458
	CD14/ml	0.00251	0.000783	0.0030
	ConA	0.00255	0.00124	0.0483
	SomAg	0.0790	0.00963	0.0000
lme_6 (Ab) *Farms B & C*	Farm C	-0.201	0.0862	0.0292
	Days exposure	0.00407	0.000737	0.0000
	CD4/ml	-0.0000989	0.0000374	0.0101
	CD8/ml	0.000381	0.000107	0.0006
	SomAg	-0.00686	0.00333	0.0429
lme_7 (ΔAb) *Sero-negative values; Farms A, B & C*	Farm B	-12.009	4.675	0.0151
	Farm C	-14.760	5.096	0.0068
	CD8/ml	0.0272	0.00997	0.0080
	SomAg	-0.621	0.276	0.0277
	TegAg	0.712	0.248	0.0054
lme_8 (ΔAb) *Farms B & C*	*Not significant*			

4.5 Discussion

Serum antibody ELISA results indicated all individuals in the cohort of heifers from farm A became infected or exposed, and positive fluke egg counts confirmed this in 10 of the 17 animals. The range of PP values in these animals at the final time point may reflect a number

of factors, including both duration and intensity of infection, since serum antibody response in cattle has been shown to correlate positively with fluke burden (Salimi-Bejestani et al., 2008). There is little evidence to suggest the cohorts from either farms B or C became infected even though *F. hepatica* was shown to be present in the lactating animals on both farms. Although two animals from farm B had PP values that exceeded the 20PP diagnostic cut off at a single time point, the values were close to the cut off value (20.3 & 24.7) and readings were negative for the remainder of the study. This suggests that these may have been false positive values resulting from the inherent variation in ELISAs. It is likely that the two cohorts on Farm B and C were not turned out to pastures that were contaminated with *F. hepatica* metacercariae in the Spring. That being said, there was a small but notable trend of increasing in antibody PP values towards the end of the grazing season for both farms B and C which may indicate a low level exposure did occur at this time. This lack of, or delay in exposure may in part be due to 2013 being a warmer and drier summer than the seasonal average (1981-2010) (Met-Office, 2013) delaying development and subsequent release of metacercariae onto the pasture during this period (April to November).

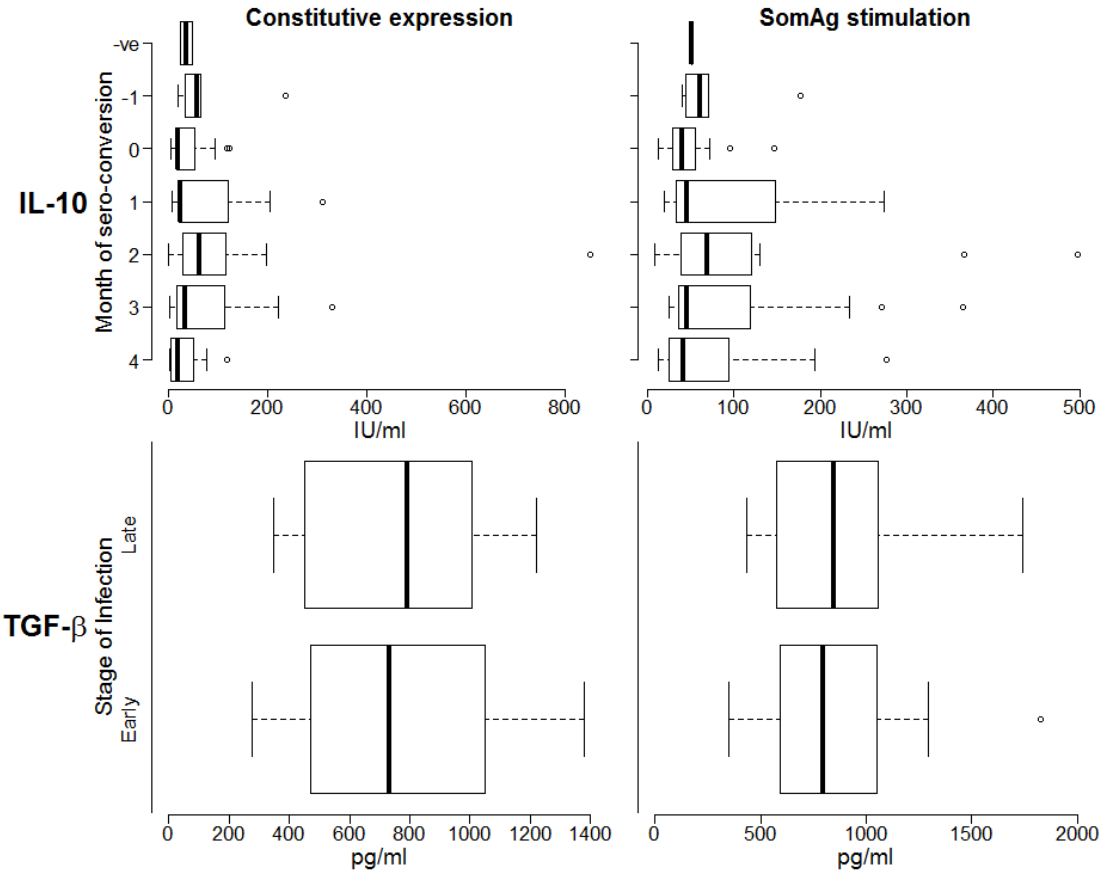

Figure 4.4: *Ex vivo* PBMC culture cytokine profiles for Farm A (2). Cytokine production (IL-10 and TGF-β) by *ex vivo* PBMCs either constitutive (left) or in response to stimulation with SomAg (right). For IL-10, outputs for 1 month prior to sero-conversion (-1) are shown separately from other sero-negative values (-ve) since infection could be present at this time point. For TGF-β, early stage infection data represents sample measurements from time points at or close to sero-conversion, with late stage infection representing the last available time point post exposure.

Eosinophil counts increased over the course of the grazing season in all three groups. There was no evidence of *F. hepatica* infection at the time of these observed increases on farms B and C suggesting it is most likely these were associated with nematode burdens, since eosinophilia is a feature common to most helminth infections (Tizard, 2013). With respect to the cohort from farm A, it is more likely this eosinophilia was due to *F. hepatica* since these animals received regular anthelmintic dosing throughout the grazing season as part of the normal farm management procedure, which is reflected by the finding that only two animals had a positive nematode egg count of 50 eggs per gram (epg) at a single time point. Furthermore, the significant positive association between Ab and SomAg-stimulated IL-5 transcription in this cohort supports the idea of a *F. hepatica* induced eosinophilia. This is consistent with previous findings of eosinophilia in *F. hepatica* infected cattle (Bossaert et al., 2000b; Ross et al., 1966). Whilst there is experimental evidence to suggest eosinophils can play a protective role in the early stages of infection in an ADCC type manner (Davies and Goose, 1981; Duffus et al., 1980; Van Milligen et al., 1999; Van Milligen et al., 1998), such activity has not been demonstrated in cattle *in vivo*. Whilst our findings provide evidence of *F. hepatica* induced eosinophilia in naturally infected cattle, these do not indicate what role these cells play in the overall immune response.

4.5.1 Ab PP values and ΔAb

Multivariable analysis confirmed the positive relationship observed between PP value (Ab) and days exposure. Conversely, following sero-conversion, change in PP value (ΔAb) was shown to be negatively associated with days exposure. This suggests that while PP value (Ab) increased over the course of infection, ΔAb represents a useful parameter with which to investigate the early stages of infection.

The positive relationships found between PP value (Ab) and eosinophil counts, Con A-stimulated IL-4 production, SomAg-stimulated IL-5 transcription, and negative association with ConA-stimulated IFN-γ production and SomAg-stimulated PBMC proliferation suggest that as *F. hepatica* infection progresses, this is associated with a polarisation of the immune response towards a type-2 response. These findings are consistent with previous experimental studies in cattle, where initial mixed proliferative, mixed inflammatory responses subside and are replaced by non-proliferative responses characterised by IL-4 production and IgG1 isotype antibody production (Brown et al., 1994; Clery et al., 1996; Clery and Mulcahy, 1998; Oldham and Williams, 1985).

Positive correlations between ΔAb and both IL-4 production and IL-5 transcription suggest these cytokines are prominent features of the early immune response following natural infection. This differs somewhat from previous findings in experimentally infected cattle,

where IL-2 and IFN-γ production have been observed in the first 2-3 weeks of infection (Clery and Mulcahy, 1998; Oldham and Williams, 1985). This finding may suggest that polarisation of the immune response in naturally infected cattle occurs from the early stages of infection, although this may also be a result of the time frame over which these animals were sampled. Animals were sampled at monthly intervals, meaning early transient mixed and/or pro-inflammatory responses may have been missed.

Although a positive relationship was found between PP value (Ab) and $CD8^+$ T-cell counts for infected animals, this relationship was also demonstrated when analysing sero-negative data. This finding may be attributed to age and maturation of the immune system rather than a direct result of *F. hepatica* infection. The same is likely to be true when considering the negative relationship found between PP value (Ab) and $WC1^+$ γδT-cell counts, as this relationship was also found in uninfected groups. $WC1^+$ γδT-cells are known to decrease in number in the peripheral blood as cattle mature (Hein and Mackay, 1991). At the start of the study, animals ranged in age from 90-377 days, and by the final sampling ranged from 310-587 days. The mean $WC1^+$ populations were 20% (6-32%) of total PBMCs at the first sampling and 6% (0.5-24%) by the final time point. In uninfected cattle, $WC1^+$ cell populations constitute approximately 15% of PBMCs in animals aged 3-12 months in age, with this number decreasing to around 5% by 3 years of age (Clevers et al., 1990). The similarity of these figures to our own findings suggests the observed decrease in $WC1^+$ PBMCs over the study period was the result of a normal physiological process.

On farm A, both *F. hepatica* and Paramphistome eggs were observed in faecal samples. The *F. hepatica* antibody-ELISA has been shown not to cross-react with common GI nematodes of cattle (Salimi-Bejestani et al., 2005b). Although this ELISA has not been evaluated for cross-reactivity with Paramphistome spp. trematodes, it has been assessed for a commercially available coproantigen ELISA that uses mouse monoclonal antibody (MM3 mAb) to detect *F. hepatica* E/S in faeces (Mezo et al., 2004). Studies comparing coproantigen ELISA results to fluke egg counts found no evidence of false positive results due to paramphistome infections (Kajugu et al., 2015), whilst a further investigation examining the specificity of the MM3 mAb via immunohistochemistry found no antigen-specific antibody binding in sections of the paramphistome *Calicophoron daubneyi* (Gordon et al., 2013). Furthermore, bovine immune responses to *C. daubneyi* are characterised by localised IFN-γ production, pro-inflammatory cellular infiltrates and $CD8^+$ T-cell mediated immunity in the rumen (Fuertes et al., 2015). This makes it unlikely that either antibody responses or the associated immune responses observed in this study were the result of Paramphistome infection.

4.5.2 *F. hepatica*-specific Proliferation

The negative relationship found between both Ab and ΔAb with SomAg specific PBMC proliferation suggests that naturally acquired *F. hepatica* infection results in a loss of proliferative responses to SomAg. Clery and Mulcahy (1998) showed that parasite specific proliferative responses occurred in conjunction with IFN-γ production over a period of 2 to 3 weeks post infection in experimentally infected calves (Clery and Mulcahy, 1998). In our study, early proliferative responses were observed only in some animals. It is likely this is partly due to the monthly gap between samplings, which limited our ability to detect transient early responses, although inherent variations in individual responsiveness and differing intensities of infection are also likely to have contributed to these differences.

Multivariable analysis revealed differences between ΔAb and PBMC stimulation to TegAg and SomAg; a negative association found with SomAg stimulated proliferation and positive association with TegAg proliferation. TegAg comes into direct contact with the host *in vivo* and may induce proliferation, particularly in the early stages of infection.

When SomAg was modelled as the response variable a significant positive association was found with ConA specific IL-4 production and $CD4^+$ T-cell counts, and a negative association with $CD8^+$ T-cell counts. When TegAg was modelled as the response variable, a negative relationship was again shown with peripheral blood $CD8^+$ T-cell counts. Collectively, these findings would support our results indicating a type-2 polarisation of the bovine immune response to naturally acquired *F. hepatica* infection. The positive relationship found between TegAg proliferation and $CD14^+$ blood monocyte counts may relate to their role as APCs within PBMC cultures. TegAg has previously been shown to induce alternative activation and increased arginase expression in naïve $CD14^+$ blood monocyte derived macrophages *in vitro* (Hacariz et al., 2011), with similar effects having been observed in both mouse macrophages and DCs (Adams et al., 2014; Hamilton et al., 2009). This may be a significant observation, highlighting the role of $CD14^+$ blood monocytes in the bovine immune response to natural *F. hepatica* infection, although without additional supportive evidence such as cytokine production, arginase expression, NO production etc. the exact nature of this relationship remains unclear.

4.5.3 *F. hepatica*-induced Immune-modulation

There is evidence from experimental infections to suggest that *F. hepatica* is capable of modulating host immune responses. SomAg has been shown to induce M2 activation of bovine macrophages in experimentally infected cattle from 12 weeks post infection (Golden et al., 2010), and TegAg has been shown to induce alternative activation in ovine, murine and human macrophages *in vitro* (Adams et al., 2014; Figueroa-Santiago and Espino, 2014; Hacariz

et al., 2011). Furthermore, recent analysis of the *F. hepatica* genome has uncovered the presence of TGF-β homologues which could play a direct role in the immuno-modulation of host immune responses (Japa et al., 2015).

It has been suggested that the parasite-driven immune modulation prevents protective immunity developing, allowing the parasite to survive within its definitive host for months or even years. There is evidence to suggest this modulatory effect may make animals more susceptible to further infection by both *F. hepatica* itself as well as other pathogens such as *S. dublin* and *M. bovis* (Claridge et al., 2012; Clery et al., 1996; Flynn et al., 2009; Garza-Cuartero et al., 2016; Vaessen et al., 1998).

Our study showed a reduction in SomAg-stimulated proliferative responses in early and late stages of infection, whilst increased ConA-stimulated IL-4 and decreased ConA-stimulated IFN-γ production indicates a polarisation of the response. This is consistent with previous studies in experimentally infected cattle where both adaptive and innate responses have been shown to be altered by presence of *F. hepatica* (Flynn et al., 2007b; Flynn et al., 2009).

In addition to this polarising effect, parasite specific immune modulation has also been shown to increase regulatory responses in the chronic stages of infection, with significant elevations in parasite specific IL-10 and TGF-β observed in experimentally infected animals (Flynn and Mulcahy, 2008b). In this study, no association was found between parasite-specific IL-10 or TGF-β production by PBMCs and infection status. IL-10 expression has been demonstrated previously in hepatic tissue taken from naturally infected cattle (Mendes et al., 2013), whilst Hacariz et al. (2009) demonstrated the presence of both IL-10 and TGF-β gene transcription in the hepatic lymph nodes of experimentally infected sheep. The absence of these cytokines in our study may therefore indicate that unlike experimental infections, these responses are either not present in natural infection, or are only present in the local immune response.

4.5.4 Conclusion

In conclusion, *F. hepatica* infection in cattle under natural field conditions resulted in the polarisation of immune responses towards a non-proliferative type-2 response as has previously been observed in experimentally infected animals. These findings therefore support ongoing work into the development of vaccines aiming to induce a protective immunity through the induction of additional ADCC-type responses, although type-2 responses to as yet unseen antigens may be important. The results also suggest that administration of vaccines prior to exposure will be necessary, as the modulatory effects resulting from natural infection may reduce vaccine efficacy. Furthermore, the apparent polarisation of the immune response supports previous work indicating that *F. hepatica* infection has wider implications for animal

health and disease control, emphasising the need for further work investigating these effects and their potential epidemiological consequences in greater depth.

4.6 Acknowledgments

We would like to thank Dr Jayne Hope (Roslin institute, Edinburgh, UK) for the kind donation of the recombinant IL-10 ELISA standards used in this study and the farmers for their co-operation and willingness to get involved with our investigation.

Chapter 5

Immune Responses of Adult UK Dairy Cattle Harbouring Chronic, Naturally Acquired Infection with *Fasciola hepatica*

5.1 Abstract

Fasciola hepatica is a common parasite of cattle globally, causing significant economic losses and compromising animal welfare. In addition to causing clinical and production limiting disease, *F. hepatica* is also important because of its ability to modulate bovine immune responses against both itself and other co-infecting pathogens.

As a result of emerging drug resistance and increasing disease prevalence in the UK and other parts of Europe there is a need to develop new and sustainable methods of disease control. Vaccination has been proposed as a potential adjunct to current control measures, with experimental trials showing potential. Little is known, however, of the bovine immune response to natural infection with *F. hepatica* or how such responses would impact on vaccination strategies. Increasing our understanding in this area, particularly with respect to the presence and character of parasite-induced immune modulation will help inform not only vaccine development, but also help to evaluate the potential implications *F. hepatica* has on host responses to co-infecting pathogens.

In this investigation we defined immune responses in chronically infected adult dairy cows in a UK dairy herd naturally exposed to *F. hepatica*. Animals (n=27) were sampled towards the end of lactation with infection status determined by parasite-specific serum antibody ELISA. Both infection status and antibody PP-values were analysed using logistic and linear regression analysis respectively against immunological parameters in the form of circulating leukocyte counts and their proliferative and cytokine responses to *in vitro* stimulation with mitogen and parasite specific antigen to characterise the immune responses associated with infection.

Our study demonstrated that cattle with chronic, naturally acquired *F. hepatica* infection display type-2 immune responses, characterised by parasite specific and ConA stimulated IL-4 production by *ex vivo* PBMCs and an absence of IFN-γ production and proliferative responses.

Seventeen cows were re-sampled following calving, with 10 animals having received treatment with triclabendazole (TCBZ) to clear infection at the start of the dry period. Treatment was found to clear infection and reduce fluke egg counts to zero in all cases. Changes in TGF-β and IL-10 production were also observed in these animals, suggesting an underlying regulatory mechanism.

5.2 Introduction

The Liver Fluke *(Fasciola hepatica)* is a parasitic trematode with a worldwide distribution. It is capable of infecting a range of vertebrate species, including livestock, particularly sheep and cattle. It is an important zoonosis in many developing countries.

Infection occurs through ingestion of infective metacercariae encysted on herbage. Once ingested, juvenile parasites excyst and migrate through the wall of the small intestine and across the peritoneum, penetrating the liver capsule and migrating through the parenchyma to reach the bile ducts where they become sexually mature, achieving patency around 10-12 weeks post infection (Dixon, 1964; Ross et al., 1966). Infection may result in clinical disease (fasciolosis), although in host species such as cattle infection is often asymptomatic. In either case, *F. hepatica* can have a profound impact on the productivity of food producing animals (Charlier et al., 2007; Howell et al., 2015; Lopez-Diaz et al., 1998).

Prevalence of *F. hepatica* in the UK dairy herd has increased in recent years (Howell et al., 2015; McCann et al., 2010; Salimi-Bejestani et al., 2005a). This is largely attributed to changes in climatic conditions favouring the external developmental stages of the parasite and its snail intermediate host *Galba truncatula*. Epidemiological models which use climatic conditions to predict *F. hepatica* prevalence have demonstrated this to be the case when compared to observed disease prevalence retrospectively, and suggest this trend will continue in the UK and Europe over the coming decades if forecasted changes in weather patterns are correct (Caminade et al., 2015; Fox et al., 2011).

Disease control is largely dependent upon the use of a small number of effective anthelmintics (flukicides), with the treatment of dairy cattle further constrained by licensing restrictions on the use of many of these products during lactation. Charlier et al. (2012) demonstrated that treatment with closantel over the dry period can be an effective method of control in such animals, with a lowering of serum antibody titres and increased milk yield observed over the course of a subsequent lactation. However, the emergence of resistant populations of parasites to flukicides resulting from their prolonged use complicates matters further. The majority of reports both in the UK and abroad have identified resistance to triclabendazole (TCBZ) (Gordon et al., 2012; Moll et al., 2000; Ortiz et al., 2013; Overend and Bowen, 1995), although a recent report has also detected the emergence of resistance to closantel in cattle (Novobilsky and Hoglund, 2015).

An additional and important feature of *F. hepatica* infection in cattle is the parasite's ability to modulate host immune responses. It is thought this is principally to allow the parasite to survive within the host, with periods of several months up to and in excess of a year recorded in untreated cattle (Andrews, 1999; Dalton et al., 2013; Ross, 1968b). In addition to prolonging parasite survival, however, this also has a more general effect on the bovine immune response

resulting in so-called bystander effects, where infected animals display an impaired ability to respond to co-infecting pathogens. Such effects have been observed in cattle co-infected with *Salmonella dublin* (Aitken et al., 1979), whilst the sensitivity of the single intradermal comparative cervical tuberculin (SICCT) test used to detect infection with *Mycobacterium bovis* is shown to be reduced in animals co-infected with both *M. bovis* and *F. hepatica* (Claridge et al., 2012; Flynn et al., 2007b). Such bystander effects have serious implications for herd health and disease control in the wider context, emphasising the importance and concern over the increasing prevalence of, and associated difficulties in controlling fasciolosis in cattle in the UK.

There is a need to develop new control strategies, and use those currently available, in a more efficient and sustainable manner, with vaccination proposed as an alternative to the current reliance on flukicide treatment. Whilst progress has been made in this field there is still considerable variation in the levels of protection reported between vaccine trials in cattle (0-72%) indicating further research and refinements are required (Toet et al., 2014). However, where vaccine-induced protection has been achieved this has been associated with an increase in IgG2 isotype antibody titres and avidity (Golden et al., 2010; Mulcahy et al., 1998). Since Estes et al. (1994) demonstrated that IgG2 production in cattle is induced by IFN-γ, this indicates type-1 immune responses are an important feature of protective vaccine-induced immunity.

Conversely, the predominant profile in unvaccinated cattle is a polarised type-2 and regulatory immune response, particularly in the chronic stages of infection. It is also known that these responses are not protective, as chronically infected cattle have been shown to remain fully susceptible to re-challenge by experimental infection (Clery et al., 1996). Epidemiological evidence shows disease prevalence increases with age, indicating animals continue to acquire infection throughout their lifetime and do not acquire immunity (Gonzalez-Lanza et al., 1989).

This non-protective, polarised immune response is thought to be the result of parasite-induced immune-modulation. *F. hepatica* produces a number of immune-modulatory molecules in both its E/S products and surface tegument. These have been shown to alter innate cellular responses, particularly macrophages and antigen presenting cells (APCs), which ultimately modify the type of adaptive immune response activated. Both bovine and ovine $CD14^+$ blood monocyte-derived macrophages have been shown to undergo alternative activation following stimulation with *F. hepatica* antigens (E/S and TegAg) *in vitro*, exhibiting decreased nitric oxide (NO) production, increased arginase-I (Arg1) activity and IL-10 production (Flynn and Mulcahy, 2008a; Hacariz et al., 2011). In mice, similar effects have been observed in both macrophages and dendritic cells (DCs) following *in vitro* stimulation with E/S antigen. Furthermore, subsequent co-culture of these altered DCs and macrophages with naïve $CD4^+$ T-cells resulted in a polarised adaptive response, including increased IL-4, IL-5 and decreased IFN-γ production

as well as increased regulatory activity in the form of IL-10 and TGF-β production and up-regulation of FoxP3 expression (Falcon et al., 2010; Guasconi et al., 2015). Similarly, in experimentally infected cattle the alternative activation of macrophages was found to be associated with increased IL-4 and IL-10 production in PBMC cultures (Flynn and Mulcahy, 2008a).

The overall effect of this parasite induced immune modulation is the progression, over the course of infection, from an initial proliferative response with mixed type-1 and type-2 cytokine production in the early stages of infection, to a polarised non-proliferative response with type-2 and regulatory cytokine production in the chronic stage. In experimentally infected cattle this is observed as a progressive decline from what is initially a strong parasite-specific proliferative response combined with IL-2 and IFN-γ production by *ex vivo* PBMC cultures which then becomes undetectable from around 5 weeks post infection onwards (Clery and Mulcahy, 1998; Oldham and Williams, 1985). The predominant immunological features present in chronic infection are adaptive responses comprised of T_H0/T_H2 cells, eosinophilia and a serum IgG1 antibody response (Bossaert et al., 2000a; Bossaert et al., 2000b; Brown et al., 1994).

Whilst parasite-induced immune modulation initiates polarisation of host immune responses; the resulting regulatory responses present in chronic infection appear to augment and sustain this effect. *Ex vivo* PBMCs taken from *F. hepatica* infected cattle show IL-10 and TGF-β production in response to E/S antigen stimulation from 4 weeks post infection onwards, with these two regulatory cytokines shown to have a direct down-regulatory effect on IFN-γ and IL-4 production, respectively (Flynn and Mulcahy, 2008b). Similarly, presence of IL-10 in *in vitro* cultures of T-cell clones isolated from chronically infected cattle resulted in the down-regulation of mRNA transcription of IL-2 and its surface receptor molecule in addition to suppressed IL-4 and IFN-γ expression. These authors also reported a suppression of proliferative responses when stimulated with Liver Fluke Homogenate (LFH) (Chitko-McKown et al., 1995).

With respect to *F. hepatica* induced bystander effects and altered responses to co-infecting pathogens, a number of significant immunological changes have been observed. In experimentally infected cattle, suppression of PBMC proliferation in response to stimulation with ConA suggests an alteration of the general immune response (Flynn and Mulcahy, 2008a; McCole et al., 1998a). More specifically, $CD14^+$ blood monocyte-derived macrophages taken from cattle co-infected with *F. hepatica* and *M. bovis* were found to undergo alternative activation with an associated reduction in IFN-γ and increase in IL-4 and TGF-β production in PBMC cultures stimulated with *M. bovis* derived PPD (Flynn et al., 2009). Similar effects have also been observed in mice infected with *Bordetella pertussis* where either co-infection with *F.*

hepatica or parenteral injection with *F. hepatica* E/S antigen has resulted in a suppression of *B. pertussis* specific IFN-γ production *in vivo* (Brady et al., 1999; O'Neill et al., 2001).

The high *F. hepatica* sero-prevalence within the UK dairy herd makes it likely that chronically infected cattle represent a substantial percentage of the overall population, particularly since treatment opportunities in these animals are limited (NOAH, 2016). Gaining a better understanding of the immune responses present in these animals and establishing whether a parasite induced immune-modulation is present will provide important information not only for future vaccine development, but also improve our understanding of the wider epidemiological implications of *F. hepatica* infection, particularly from the perspective of bystander effects on co-infecting pathogens. In this study we investigated the immune responses in UK dairy cattle with naturally acquired chronic *F. hepatica* infections, in order to inform vaccine development and to determine whether there is evidence of immune modulation under such conditions.

5.3 Materials and Methods

5.3.1 Study Design

Samples were taken over the course of the 2014 grazing season (May to October) from adult dairy cattle (n=27) at a local dairy farm in Warrington, Cheshire. Animals were initially sampled as they reached the end of their lactation, in the month before drying off. Serum and EDTA treated whole blood was collected via jugular venepuncture and faeces were collected rectally. Bulk milk tank (BMT) samples were collected at each visit to monitor the infection status in the milking herd over this period (Salimi-Bejestani et al., 2005a). It is worth noting this farm had historically high BMT ELISA results over the previous 12 months. Treatment history on this farm consisted of a single dose of oxyclozanide administered to the milking herd one year previously.

During this study, all animals were kept under normal farm management conditions, staying at pasture throughout the grazing season. At the first sampling, infection status was evaluated by both serum antibody ELISA and fluke egg counts. This was fed back to the farmer to allow infected animals to be treated with triclabendazole (TCBZ) at the start of the dry period at the recommended dosage (12mg/Kg bodyweight).

To investigate the effects of treatment, 17 of the original 27 animals were re-sampled post-calving. Ten of these animals had received treatment with TCBZ at drying off (table 5.1). Of the seven untreated animals, six were sero-negative at the initial sampling, whist the one remaining animal was sero-positive but had received no treatment because the time from the initial sampling to the expected calving date being less than 48 days, the minimum withdrawal period for use of TCBZ in non-lactating dairy cattle in the UK (table 5.1).

Table 5.1: Summary of treatment and infection status for twenty-seven cows sampled at drying off. Infection status indicates the sero-status of animals at the first sample point (drying off).

Infection status	Treatment	First sample (drying off; n=27)		Re-sample (post-calving; n=17)		
		Number	FEC positive	Number	Sero-positive	FEC positive
Sero-positive	Treated	18	11	10	8	0
	Untreated	1	0	1	1	1
Sero-negative	Untreated	8	0	6	3	1

5.3.2 Infection Diagnosis

Clotted serum tubes were centrifuged at 2,000g for 5 min to separate serum. Serum was aliquoted and stored at -20°C until tested. *F. hepatica*-specific serum antibody was measured by ELISA with a diagnostic cut-off of 20PP at a quoted diagnostic sensitivity and specificity of 95% and 99% respectively (Salimi-Bejestani et al., 2005b) (Section 2.6).

Coprological analysis was performed using a Flukefinder® egg sedimentation kit (www.flukefinder.com) on 3g faecal samples, and nematode faecal egg counts were assessed using a standard McMaster's technique (Thienpont et al., 1986).

5.3.3 Leukocyte Enumeration and PBMC Purification

Total leukocyte enumeration was performed on EDTA treated whole blood following 1:20 dilution in a 1% gentian violet, 2% acetic acid solution to stain nuclei and lyse erythrocytes. Prepared samples were loaded and counted on a 102 FastRead™ multi chamber haemocytometer (immune systems, Paignton UK) from which total leukocyte counts/ml peripheral blood were calculated (Section 2.11). Differential cell counts for eosinophils (Eϕ), neutrophils (Nϕ), lymphocytes (Lϕ) and monocytes (Mϕ) were calculated as a percentage of total leukocyte counts following microscopic examination of thin blood smears prepared with Wright's stain (Sigma-Aldrich, St. Louis USA) following manufacturer's recommendations (Section 2.11.1).

One colour indirect immunofluorescence was performed on Peripheral Blood Mononuclear Cells (PBMCs) as previously described (Rosbottom et al., 2007). PBMCs were isolated from EDTA treated whole blood in a lateral flow hood under sterile conditions using Optiprep™ (Sigma-Aldrich, St. Louis USA) following manufacturer's recommendations (Section 2.8); briefly, Optiprep was mixed with blood at a concentration of 1.3ml per 10ml of blood in a falcon tube and layered with 1ml 20mM tricine-buffered saline and centrifuged at 1,000g for 35 min at 20°C with the brake off. The middle aqueous PBMC containing layer was harvested and transferred to a fresh collection tube, washed in 20ml PBS with 0.1% EDTA (Lonza, Bazel Switzerland) then centrifuged at 350g for 8 min at 20°C. Supernatants were discarded and haemolysis performed by re-suspending cells in 2ml of 0.9% NH_4Cl and agitated for 1 min at room temperature after which samples were washed in 20ml PBS EDTA and spun at 150g for 8

min at 20°C a further 2 times. Purified PBMCs were then re-suspended in RPMI supplemented with 10% heat inactivated foetal calf serum (Sigma-Aldrich, St. Louis USA) and penicillin and streptomycin at 100µg/ml (Sigma-Aldrich, St. Louis USA) for enumeration using a 102 FastRead™ multi chamber haemocytometer with Trypan blue solution to exclude dead cells.

For flow cytometry, samples were pipetted in duplicate into 96-well round bottomed mixing plates (5 x 10^5 cells/well) and centrifuged at 500g for 5 min to pellet cells. Supernatants were discarded and cells were re-suspended and incubated with primary mouse anti-bovine cell surface receptor monoclonal antibodies diluted in PBS with 1% BSA, 0.1% sodium azide at concentrations outlined in table 5.2, 25µl per well, and incubated on ice for 10 min. Cells were then washed twice consecutively with PBS/BSA/Azide, 100µl/well, centrifuged at 500g for 5 min and supernatants discarded after each wash/spin cycle. Samples were then incubated with secondary FITC-conjugated goat anti-mouse IgG monoclonal antibody (Sigma-Aldrich, St. Louis USA) at a 1:200 dilution, 25µl per well, in the dark on ice for 10 min. Samples were then washed as described previously prior to fixation with 1% neutral buffered formalin, 100µl/well, in the dark on ice for 10 min. A final wash step was performed prior to storage in PBS with 0.1% BSA/sodium azide, 200µl/well, overnight at 4°C ahead of flow cytometry.

Table 5.2: Primary monoclonal antibody dilutions for indirect immunofluorescence staining of PBMCs. Dilution is expressed as a ratio of antibody stock solution to PBS with 1% BSA, 0.1% sodium azide.

Cell type	Antibody	Dilution
$CD4^+$	IL-A11	1:10
$CD8^+$	(Serotec MCA837GA) CC-63	1:100
$WC1^+$	CC-15	1:10
$CD14^+$ monocytes	CCG-33	1:10

Samples were analysed using a MACSQuant® analyser (Miltenyi Biotech Ltd.). PBMCs were selected for by gating on forward and side scatter channels, following which cell surface receptor expression was measured through FITC-specific fluorescence (Section 2.11.2). Total cell phenotype counts/ml in peripheral blood were then calculated from their respective percentage value of the total circulating lymphocyte count.

5.3.4 *In vitro* PBMC Culture and *F. hepatica* Antigen Preparation

PBMC proliferative and cytokine responses were measured in response to stimulation with mitogen and *F. hepatica* antigens. Adult *F. hepatica* tegument and somatic antigen fractions were prepared through adaption of previously described methods (Hamilton et al., 2009; Hillyer, 1980; Oldham and Williams, 1985); briefly, live adult fluke collected from infected livers were incubated in RPMI (Sigma-Aldrich, St. Louis USA) with 25µg/ml genticin (Invitrogen Life Technologies, Grand Island USA) overnight at 37°C in 5% CO_2 to purge caecal contents,

then washed three times in ice cold D-PBS (Sigma-Aldrich, St. Louis USA). Following this Tegument antigen (TegAg) was prepared by placing the fluke in D-PBS with 1% Nonidet P-40 (BDH Chemicals, Poole UK) at a volume of 1ml/fluke and rocked for 1 hour at 4^{o}C. The tegument containing supernatant was then harvested and Pierce® Detergent Removal spin columns (Thermo Fisher Scientific, Waltham USA) used to remove Nonidet P-40 detergent from the antigen preparation following manufacturer's recommendations (Section 2.5.2).

Somatic antigen (SomAg) was then prepared from tegument depleted specimens. Fluke were initially washed three times in ice cold D-PBS and snap frozen overnight at -80^{o}C and homogenised in D-PBS. This mixture was transferred to a 50ml falcon tube and diluted to a concentration of 0.5ml/fluke in D-PBS and left to stand overnight at 4^{o}C. The supernatant was pipetted off and centrifuged at 12,000g for 30 min at 4^{o}C then filter sterilized with a 0.2µm-pore filter (Section 2.5.3). Protein concentration of both antigen fractions was determined using the Coomassie Plus (Bradford) assay and bovine serum albumin standards (Thermo Fisher Scientific, Waltham USA) and stored at -80^{o}C ahead of use.

To measure proliferation, PBMCs were incubated with the mitogen concanavalin A (ConA, 5µg/ml), *F. hepatica* SomAg and TegAg (25µg/ml) and as unstimulated medium controls as described previously (Williams et al., 2000). Briefly, PBMC samples were adjusted to a concentration of 2 x 10^6 per ml in RPMI with 10% FCS and 100µg/ml penicillin and streptomycin. Triplicate samples were placed into 96-well U-bottomed tissue culture plates (Corning Life Sciences, Corning USA) and incubated for 5 days at 37^{o}C, 5% CO_2, with 2 x 10^5 PBMCs and 200µl culture media per well. Cells were pulsed with 1µCi of [H^3] tritiated thymidine for the final 5 hours of incubation then harvested onto glass filter mats and embedded in scintillation wax (Perkin Elmer, Boston USA). β-particle emission counts were measured using a MicroBeta^2 plate counter (Perkin Elmer, Boston USA), with mitogen and antigen specific proliferation expressed as a stimulation index (SI), which is defined as the fold increase in β-particle decay with reference to medium controls. Where SI for the ConA positive control was <2, sample values were excluded from analysis.

Cytokine expression was quantified based on PBMC culture supernatant concentration following incubation with ConA (5µg/ml), *F. hepatica* SomAg (25µg/ml) and medium controls. Samples were placed into 24-well flat bottomed tissue culture plates in a total volume of 2ml RPMI with 10% FCS and 100µg/ml penicillin and streptomycin (VWR, Radnor USA) and incubated for 48 hours at 37^{o}C, 5% CO_2, 2 x 10^6 PBMCs/well. IFN-γ expression was measured using a commercially available sandwich detection ELISA following manufacturer's protocols (MCA5638KZZ, AbD Serotec, Raleigh USA), whilst IL-4 and Interleukin-10 (IL-10) expression were measured by following previously defined protocols for the detection of these cytokines using a bovine specific sandwich detection ELISAs (Hope et al., 2005; Kwong et al., 2002).

Transforming growth factor-β (TGF-β) expression was measured, following acid treatment to convert TGF-β to its bio-active form, using a commercially available sandwich detection ELISA for human TGF-β shown previously to be cross-reactive with the bovine analogue (Abbott et al., 2005) following manufacturer's recommendations (TB196 Technical Bulletin, Promega, Madison USA).

5.3.5 Statistical Analysis

Measurements taken from the initial sampling towards the end of lactation (prior to calving) for all 27 animals were analysed to investigate the effect of chronic infection with *F. hepatica* on host immune responses. Univariable logistic regression was used to examine the relationship between sero-positivity (Y) and explanatory variables (x) i.e. age, worm egg counts and immunological parameters including leukocyte counts, PBMC proliferative and cytokine responses. Additionally, univariable linear regression was also used to analyse the relationship between antibody PP values (Y) and these explanatory variables (x).

The effects of treatment with TCBZ on animals after calving were analysed using non-parametric tests rather than assuming a normal distribution. The seven untreated animals sampled post calving were also included in this analysis to identify potential immunological changes associated with calving (Oliveira and Hansen, 2008; Raghupathy, 1997; Wegmann et al., 1993). Wilcoxon ranked sum tests were used to determine differences in measurements from the same animals at each time point as a paired analysis, whilst Mann-Whitney U tests were used to compare treated and untreated groups at each time point. A post-hoc Bonferroni correction was performed to compensate for these multiple test comparisons.

In addition to immunological parameters, data was also collected from the Cattle Information Service (CIS) on the production parameters for each of the 27 individuals sampled in the lactations preceding and following sampling to investigate the potential effects of fluke infection and/or treatment intervention on animal productivity. One animal was culled shortly after the first sampling and subsequently excluded from this part of the analysis.

Multivariable linear regression was used to investigate the effects of *F. hepatica* infection on productivity, with milk yield, protein and butterfat content and calving interval assessed as response variables (Y). These were compared against multiple explanatory variables (x) simultaneously, with antibody PP value and worm egg counts modelled alongside additional parameters including signalment and seasonality to assess the relationships between these parameters using data from the lactation immediately before the first sample was collected and the lactation immediately following collection of the second sample. For lactation data recorded post sampling (and calving), treatment status was also included as an explanatory variable to assess whether this had any impact, whilst change in antibody PP-value (ΔAb)

between time points was used in preference to pre- and post-calving/treatment values to reduce model complexity. Prior to this analysis, a correlation matrix was applied to the dataset to identify covariates, and any explanatory variables with a correlation coefficient (R) >0.7 were not included in the same model analyses. Stepwise AIC selection was then used to produce final parameterised model selections, which were checked for linearity and goodness of fit using residual and qq-normality plots.

5.4 Results

Antibody PP values from monthly BMT samples collected over the course of the study did not change significantly from month to month. The mean of the PP value for the six monthly BMT samples was 88 (SD ±5.5).

Of the 27 animals sampled, 19 were found to be seropositive at drying off (table 5.1). Positive worm egg counts were observed in 11 of the 19 antibody positive animals, whilst all seven sero-negative animals were fluke egg count negative (table 5.1). Eighteen seropositive animals were treated with TCBZ; one animal was not treated due to a lack of time between sampling and expected calving date. Cows were kept at pasture between drying off and calving. Seventeen of the initial 27 cows were re-sampled after calving. All ten previously infected animals were found to have negative fluke egg counts following treatment with TCBZ (table 5.1; figure 5.1a). Of the seven untreated animals, two animals had positive fluke egg counts at the second sampling, one of which was the previously infected but untreated animal (table 5.1; figure 5.1a). Fluke egg counts were low in all positive samples, with counts at or below 2 eggs per gram of faeces. Similarly, nematode egg counts were also low; only three samples had positive counts, all of which were pre-calving with none exceeding 100 eggs per gram.

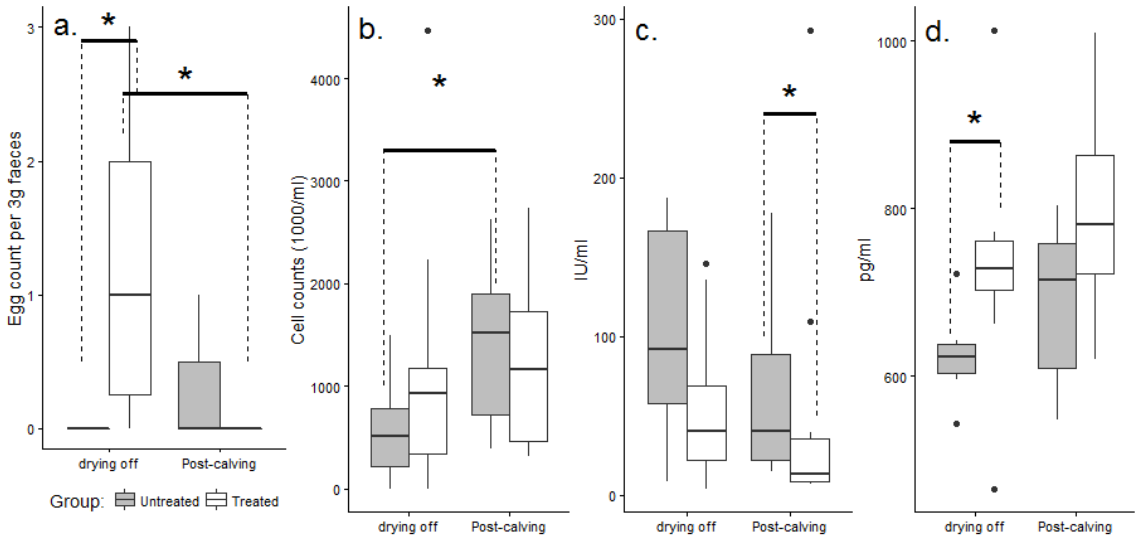

**Figure 5.1: Observed effects of calving and treatment with TCBZ on (a.) fluke egg counts, (b.) peripheral blood eosinophil counts, and (c.) constitutive IL-10 production by PBMC and (d.) TGF-β production by PBMCs. ***denotes a statistically significant difference (p<0.05)

At re- sampling, antibody PP-values decreased in 7 of the 10 treated animals, although this was not statistically significant (figure 5.2); these antibody values remained above the diagnostic cut-off value (20PP) in eight animals sampled 9-16 weeks post treatment. Three of the 6 previously sero-negative animals were found to have sero-converted post-calving (figure 5.2).

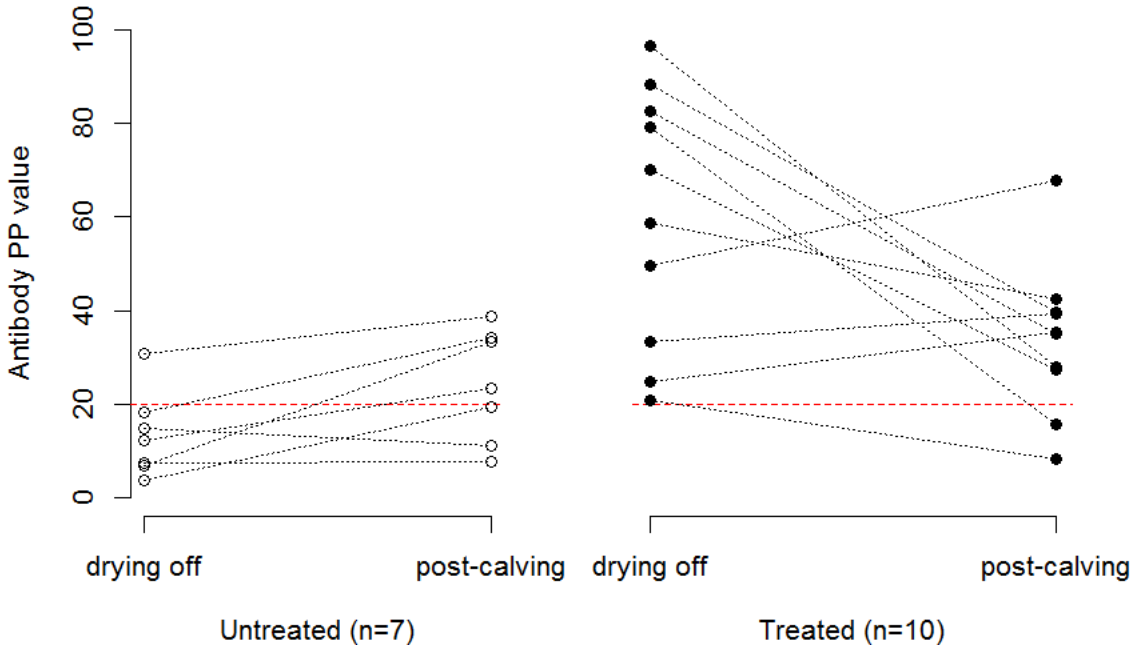

Figure 5.2: Antibody PP values for treated and untreated animals at initial sample (drying off) and re-sample (post-calving). Treated animals received a single dosing with TCBZ (12mg/Kg) over the dry cow period. (---) denotes ELISA diagnostic cut-off (20PP).

Univariable logistic and linear regression analysis are summarised in table 5.3. A significant positive relationship was demonstrated between *F. hepatica* antibody PP values and fluke egg counts, and both ConA and SomAg specific IL-4 production (figure 5.3). A negative relationship was found with both peripheral blood lymphocyte and monocyte counts. Similarly, logistic regression analysis found that infected (sero-positive) animals had significantly lower $WC1^{+}$ γδT-cell counts in peripheral circulation than uninfected (sero-negative) animals. Additionally, infection status was found to be associated with increasing animal age when analysed by either regression analysis (figure 5.3).

Following treatment with TCBZ, significant differences were found in immune responses both within and between treated and untreated groups. An increase in eosinophil counts was observed in untreated animals between the first and second sampling (p=0.03) (figure 5.1b), whilst further differences were found between the two groups in constitutive expression of both IL-10 and TGF-β. IL-10 expression was found to be higher in untreated versus treated animals post calving (p=0.04) (figure 5.1c). TGF-β expression higher in infected versus uninfected animals prior to drying off (p=0.02) (figure 5.1d).

The relationship between productivity and *F. hepatica* infection was examined; a significant positive relationship was found between milk yield values (both daily and complete lactation

data) and PP value at drying off (table 5.4), with the total lifetime yield and point at which the samples were taken through the grazing season (month sampled) also showing significant positive correlations with milk yield. No relationship was observed between milk yields and either infection status or TCBZ treatment when analysing production data from the lactation following sampling (data not shown).

Table 5.3: Immune parameters of chronic infection (univariable analysis). Statistically significant results ($p<0.05$) displayed only. [] indicates antigen/mitogen stimulation

Response variable (*Y*) [regression analysis]	Explanatory variable (*x*)	Coefficient value (β)	Standard error (SE)	P-value
Sero positivity [logistic]	Age (days)	0.003	0.001	0.045
	$WC1^+$/ml	-0.009	0.004	0.049
Antibody titre [linear]	Age (days)	0.022	0.007	0.006
	Fluke egg counts (3g)	8.536	4.132	0.049
	Lϕ/ml	-0.006	0.003	0.039
	Mϕ/ml	-0.07	0.025	0.01
	$WC1^+$/ml	-0.071	0.034	0.045
	IL-4 [ConA]	0.224	0.085	0.02
	IL-4 [SomAg]	4.346	1.695	0.023

5.5 Discussion

Nineteen out of 27 cows tested were found to be sero-positive for *F. hepatica*-specific antibodies, suggesting that a high proportion of cattle in the milking herd were infected with fluke which was reflected in the high PP values observed in BMT samples tested over the course of the study. This is consistent with previous findings that demonstrate a positive correlation between herd seroprevalence and BMT PP values (Salimi-Bejestani et al., 2005a). The fact 3 of the 5 cows which were sero-negative at drying off subsequently sero-converted over the dry period suggests transmission was occurring during this time.

Comparison of the two diagnostic tests used suggested that the Flukefinder® kit had half the sensitivity of the serum antibody ELISA, since at the initial sampling 11 of the 19 seropositive animals were also fluke egg count positive. Conversely, all ten treated animals were fluke egg count negative when tested for the second time, 9-16 weeks post treatment and 8 of these 10 animals had antibody PP values above the diagnostic cut-off (20PP). This demonstrates the greater specificity of fluke egg counts compared with serum antibody ELISA in the context of historic infections. It is also possible however that re-infection over the dry period also contributed to the antibody PP values in these animals remaining elevated, since there was evidence of infection in previously sero-negative animals at re-sampling. Nonetheless, previous studies have shown fluke-specific serum antibody titres remain elevated in cattle for several months following treatment with TCBZ (Castro et al., 2000), with serum samples remaining positive for at least 11 weeks post treatment when evaluated using the ELISA described in this study (Salimi-Bejestani et al., 2005b). Although we encountered this effect in post treatment

samples, at the time of initial sampling, prior to calving, no cows had received any form of fluke treatment for over a year. This would suggest that seropositive values in this instance were indicative of current infection, particularly since fluke eggs were also observed. The relative sensitivity and specificity of fluke egg counts and serum antibody ELISAs reported here are consistent with findings from previous studies (Anderson et al., 1999; Charlier et al., 2008b; Rapsch et al., 2006).

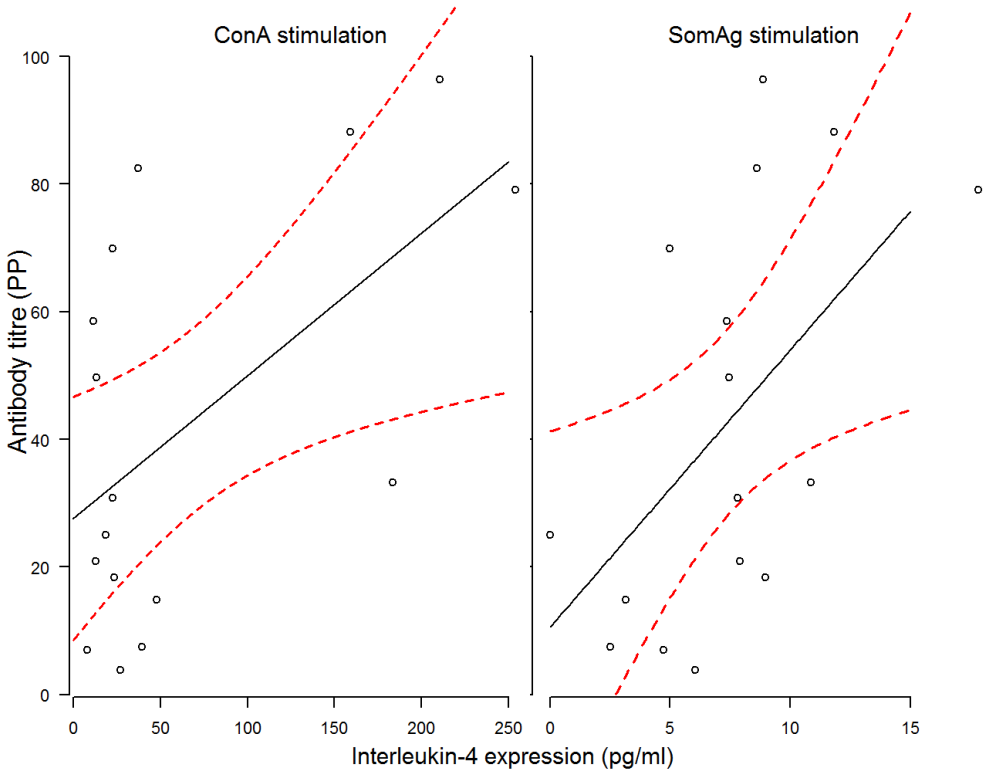

Figure 5.3: ConA and *F. hepatica* somatic antigen stimulated IL-4 expression in PBMCs versus fluke antibody titre at initial sampling (drying off). (---) denotes 95% confidence interval for fitted regression lines.

The positive correlation between antibody PP value and both ConA and parasite specific IL-4 production suggests a type-2 immune response in naturally acquired, chronic infection. This is consistent with previous findings where type-2 immune responses persist in chronic fluke infections, but do not confer any protective effect (Clery et al., 1996).

Table 5.4: Relationship between animal productivity and infection status (multivariable analysis). Only multivariate models with statistically significant results relating to parasitism (i.e. Antibody titres, worm egg count or treatment with TCBZ [p<0.05]) are displayed.

Response variable (Y)	Explanatory variable (x)	Coefficient value (β)	Standard error (SE)	P-value
Lactation yield (litres)	Month sampled	265.02	119.87	0.038
	Lifetime yield (litres)	0.029	0.014	0.048
	Antibody titre (PP)	17.886	7.803	0.032
Daily yield (litres)	Sample	0.864	0.385	0.036
	Lifetime yield (litres)	0.0001	0.00004	0.059
	Antibody titre (PP)	0.057	0.025	0.034

Lymphocyte, $WC1^+$ γδT-cell and monocyte peripheral blood counts were all found to be lower in infected versus uninfected animals. One possible explanation for this could be parasite induced anaemia, since total leukocyte counts have been found to be negatively correlated with infection status (Aitken et al., 1979). Alternatively, these decreases could indicate sequestration of these cell types in the hepatic tissue and/or lymph nodes as is observed at post-mortem in infected cattle (Rahko, 1969).

The increase in eosinophil counts post calving in untreated animals is likely to indicate these animals are becoming infected with *F. hepatica*, since eosinophilia is a common feature during the initial migratory stages of the parasite (Bossaert et al., 2000b), particularly since we also observed increases in antibody PP values in these animals.

Differences in TGF-β and IL-10 expression, observed in treated versus untreated animals pre and post treatment/calving respectively, could indicate evidence of the underlying regulatory mechanisms of immune modulation present in natural infection; in cattle sampled at drying off TGF-β expression was higher in sero-positive versus sero-negative cattle suggesting this cytokine may represent an important regulatory response in chronic infection. Elevated IL-10 expression in untreated versus treated animals post calving could suggest this cytokine has a more prominent regulatory role in the earlier stages of infection, since parasite transmission was occurring during this time. It should be noted, however, that these differences are detected in unstimulated cultures rather than in response to *F. hepatica* antigen stimulation as is the case in previous work on experimentally infected calves where TGF-β was found to be more prominent earlier in infection compared with IL-10 (Flynn and Mulcahy, 2008b). This study also demonstrated that IL-10 was predominantly responsible for suppression of parasite specific IFN-γ production, whilst TGF-β was found to suppress both IFN-γ and, to a lesser extent, IL-4 production, which would fit with our observations of a polarised non-proliferative type-2 response in the chronic stages of naturally acquired infection.

The positive correlation found between animal age and antibody PP value is similar to a positive relationship demonstrated previously between animal age and disease prevalence when established through fluke egg counts (Gonzalez-Lanza et al., 1989). This may be due to the limited use of flukicides in the milking herd on this farm. Furthermore, since serum IgG antibody titres have been shown previously to correlate with fluke burden (Salimi-Bejestani et al., 2005a), these findings could suggest that older animals harbour larger parasite burdens as a result of continual exposure and re-infection.

Beneficial effects of treatment on milk production have been reported for other flukicides (Charlier et al., 2012; Khan et al., 2011; Randell and Bradley, 1980). However, in this investigation we found no effect of TCBZ treatment on milk yield. This may be due to a lack of

power resulting from the small sample size in this part of the study or differences between farm management systems or parameters measured.

The economic effects of *F. hepatica* are well established, with a number of studies demonstrating a negative association between productivity and infection (Charlier et al., 2007; Howell et al., 2015; Kuerpick et al., 2012; Lopez-Diaz et al., 1998). Our finding of a positive correlation between milk yield in the lactation before sampling and antibody PP value at drying off is therefore unexpected. It is possible this relationship was an artefact of the relationship between animal age and antibody titre, although this should be accounted for through the inclusion of lifetime yield in the multivariate analysis, as this is a strong covariate of age. Similarly, whilst the effects of pregnancy and the high energy demands of early lactation have been controlled for where possible through sampling all animals in the same stage of production, these factors could still account for some residual effects. Since antibody titre was measured towards the end of lactation, preceding milk yield data should be considered retrospective. As a result, causality cannot be assumed; these findings could indicate that animals producing higher milk yields are more susceptible to infection. From a biological perspective this would make sense since animals producing greater milk yields would invest a larger proportion of their physiological resources into this process to the detriment of other systems, potentially increasing susceptibility to infection. This finding could also indicate an effect of selective breeding, as immune responses and yield are considered to be negatively linked genetically (Rauw, 2012). Most studies to date examining the impact of *F. hepatica* infection on dairy production have focussed on production at the herd level (Charlier et al., 2007; Howell et al., 2015; Kuerpick et al., 2012). Our findings do not necessarily contradict these; although we showed infected animals produced larger quantities of milk, infection could be preventing these animals from reaching their full potential, which at a herd level would show as reduced milk yields compared to fluke negative farms. To date, only one other study has looked into the impact of *F. hepatica* on dairy production in individual animals in Galicia, Northern Spain (Mezo et al., 2011). This study found animals with high *F. hepatica* antibody titres produced approximately 2Kg less milk per day compared to sero-negative animals, although no differences in yield were found between sero-negative animals and between those with low levels of circulating antibody. Whilst inconclusive, we feel these findings represent a notable and previously undescribed feature of the dynamics of *F. hepatica* infection in UK dairy cattle on productivity at the level of the individual, and thus warrant further investigation.

In conclusion, our findings suggest that animals naturally exposed to and chronically infected with *F. hepatica* have an immune response characterised by increased IL-4 production and an absence of IFN-γ. This suggests a type-2 response was present in these animals, which is

consistent with previous experimental findings (Brown et al., 1994; Clery et al., 1996; Mendes et al., 2013).

5.6 Acknowledgements

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Chapter 6

General Discussion

Fasciola hepatica is an important parasite globally in terms of its impact on livestock welfare and production, and due to its status as a re-merging zoonosis (WHO, 2010). Control is becoming increasingly difficult due to the emergence of drug resistance, particularly to TCBZ as reported both in the UK and abroad (Brockwell et al., 2014; Gordon et al., 2012; Moll et al., 2000; Ortiz et al., 2013). Disease prevalence and incidence is increasing due to changing climatic conditions that favour development and survival of the external developmental stages of the parasite and its intermediate snail host (Caminade et al., 2015; Pritchard et al., 2005). There is therefore a need to develop a more holistic approach to its control, with reduced reliance on anthelmintics, increased uptake of alternative and adjunctive control measures such as vaccination and a greater emphasis on the use of risk forecasting and diagnostics to better inform these control strategies. In this thesis, we have presented work contributing specifically to diagnosis through the validation of a composite sample analysis to identify *F. hepatica* infection in groups of cattle (Chapter 3) which will help to promote more sustainable disease control programmes.

With respect to vaccines, it is clear from the literature much work is still needed before a workable commercial vaccine becomes available (Molina-Hernandez et al., 2015; Toet et al., 2014). However, in light of the widespread emergence of drug resistance it is essential that vaccine development continues uninterrupted if a situation is to be avoided where disease and production losses cannot be prevented, as is the case in some parts of the world with multi-drug resistant nematode infections (Bartley et al., 2004; Wrigley et al., 2006). Against this backdrop of production limiting multi-drug resistance, the enthusiasm for, and uptake of the recently developed commercial vaccine for *Haemonchus contortus* in Australia has proven the potential impact vaccination can have in parasite control programmes (Smith et al., 2015). A number of factors need to be taken into account if such success is to be replicated in the development of a commercially available vaccine against *F. hepatica*. Previous investigation of *F. hepatica* vaccination application using stochastic mathematical modelling techniques has determined that a protective effect must be induced in at least 90% of animals for this to be an effective control strategy (Turner et al., 2016). This was derived from parameters relating to both parasite survival and development on pasture, and through consideration of the heterogeneity of host susceptibility. In terms of the latter an understanding of the types of immune response present in cattle to both vaccination and natural challenge is important. In this thesis we present information regarding such bovine immune responses to natural

infection (Chapters 4 & 5) which ultimately contributes to vaccine development programmes and our understanding of herd health more generally.

6.1 The Application of Composite Fluke Egg Counts (Chapter 3)

In Chapter 3, we showed a validation for the use of composite sample faecal egg counts as a diagnostic tool for identifying *F. hepatica* infection in cattle at the group/herd level using a standard sedimentation technique. This test has been validated previously in sheep to identify both infection and TCBZ resistance through fluke egg count reduction post-treatment (Daniel et al., 2012). Whilst available as a diagnostic service at UK government surveillance laboratories (www.apha.gov.uk), there is currently no published information available concerning its sensitivity etc. in cattle. Our validation provides useful information as to the relative sensitivity of composite analysis when compared with individual fluke egg counts, indicating that as a method of diagnosis it is as sensitive as performing multiple individual sedimentation analyses. This finding is of particular relevance to beef and non-lactating dairy cattle, as in these animals there is no alternative method of identifying infection at the herd/group level aside from multiple individual sample analyses. These findings will therefore give clinicians greater confidence in the results of the composite sample analysis, and encourage its use whilst emphasising the importance of egg counts and diagnostics generally in sustainable parasite control programmes.

Further validation in the case of bovine composite sample analysis may be warranted, particularly to determine absolute test sensitivity and specificity. Previous work investigating fluke egg count methods has achieved this by seeding negative faecal samples with known quantities of fluke eggs to give sensitivity in terms of eggs per gram of faeces (Conceicao et al., 2002). Such a validation may be an oversimplification, however, since factors such as faecal content and composition which could affect variation between samples are not taken into account. A more useful investigation would be to compare composite sample analysis with evidence of infection at post-mortem. This would be a relatively simple study to conduct through access to abattoirs, and would allow sensitivity to be defined in terms of infection status whilst also allowing a more robust evaluation of test specificity, which is generally assumed to be 100% provided no treatment interventions have been administered (Charlier et al., 2014).

Beyond this, further work to determine the usefulness of composite sample analysis as an indicator of herd prevalence would help to validate its use in farm level surveillance. Our study showed a positive relationship between group prevalence and composite fluke egg counts. Whole herd sampling to determine true herd-prevalence would allow us to further establish this relationship and to determine how many samples are required to be representative of

herd level infection as has been done previously in sheep (Daniel et al., 2012). Similar validation has been done previously with the BMT antibody ELISA in lactating dairy cattle, which was found to have a diagnostic sensitivity of 96% where >25% of the lactating herd were infected (Salimi-Bejestani et al., 2005a). Furthermore, assessment of the effects of both seasonality and repeated sampling may also provide valuable information as to the specific nature of the effects these factors may have on the sensitivity of composite sample analysis (Honer, 1965a, b).

6.2 Bovine Immune Responses to Natural Infection with *F. hepatica*

Chapters 4 and 5 examined the immune responses of animals on commercial UK dairy farms to natural infection with *F. hepatica*. These two studies defined the immune responses of cattle exposed to infection for differing lengths of time, namely primary exposure in replacement dairy heifers and chronic infection in adult cattle. Both groups were found to have non-proliferative type-2 immune responses following natural challenge. This was characterised by a presence of IL-4 production in *ex vivo* PBMC cultures, a strong parasite-specific IgG antibody response and eosinophilia. Furthermore, the absence or down-regulation of IFN-γ associated with infection in combination with an absence of IL-2 transcription and proliferative responses in *ex vivo* PBMC cultures also indicate a polarisation of the immune responses in natural infections. This corroborates findings from previous experimental infection studies where polarisation towards a type-2 response has been shown to be a common feature of both ruminant and murine immunity (Bossaert et al., 2000a; Bossaert et al., 2000b; Flynn et al., 2010). This is important, as the amount of information obtainable from experimental infections is ultimately much greater due to the increased control over, and knowledge of, intensity and duration of infection.

The increased IL-4 production (Chapters 4 and 5) and decreased in IFN-γ production (Chapter 4) observed in ConA-stimulated *ex vivo* PBMC cultures associated with *F. hepatica* infection indicates a polarisation of the general immune response occurs in naturally infected animals following primary exposure, and that this is sustained into the chronic stages of infection. This has implications for vaccine use, as it is likely these infected individuals will not respond to the vaccine in the same way as previously uninfected cattle. Furthermore, parasite-induced modulation of the host immune system has additional implications in terms of animal health, particularly with respect to susceptibility to, and control of, co-infecting pathogens (Claridge et al., 2012; Vaessen et al., 1998). The demonstration of this modulatory effect in naturally infected UK dairy cattle supports previous immunological and epidemiological evidence showing a link between *F. hepatica* infection and the diagnosis and control of *M. bovis*, with *F. hepatica* induced modulation suppressing IFN-γ production and impairing the macrophage

responses required for a positive diagnosis of *M. bovis* using the single intradermal comparative cervical tuberculin (SICCT) test (Claridge et al., 2012; Flynn et al., 2009; Garza-Cuartero et al., 2016).

One additional but important finding was the absence of a clearly defined parasite-specific regulatory response following natural infection. Neither IL-10 nor TGF-β production was found to be associated with primary *F. hepatica* infection, whilst changes were only observed in unstimulated PBMC cultures rather than in response to stimulation with parasite-specific antigen in chronically infected cattle. These findings differ from previous work in experimentally infected cattle, where parasite-specific IL-10 and TGF-β production has been observed in *ex vivo* PBMC cultures (Flynn and Mulcahy, 2008b). Regulatory responses have been observed in naturally infected cattle at the level of the local immune response (Mendes et al., 2013), suggesting the inability to detect these responses in PBMCs in our study indicates a difference in the magnitude of these responses when compared to experimental infections.

In terms of future work, analysis of archived serum samples may help to provide additional information on the type of immune responses present, specifically quantifying IgG2 versus IgG1 antibody isotype titres. Similarly, clinical biochemistry (serum GGT and GLDH levels) could also provide additional information regarding severity or intensity of infection (Bossaert et al., 2000a; Taylor et al., 2016), although care would be needed with interpretation, as these are not specific to *F. hepatica*.

Whilst cytokine and proliferative PBMC responses and characterising changes in leukocyte phenotypes are useful independently of one another, technological advances and increasing availability of bovine specific reagents such as anti-bovine cell surface receptor and cytokine antibodies in addition to an increasing array of fluorochrome conjugates could allow more integrated approaches in future. Development of multiple colour flow cytometry to facilitate the identification of both cell surface receptors, including secondary markers like FoxP3 as well as presence of intracellular cytokine production would allow the identification of cell phenotypes responsible for parasite specific and general immune responses. Similarly, intracellular staining with CFSE or the use of proliferative markers such as Ki-67 could also be incorporated to assess proliferation responses and used in conjunction with cell surface staining and/or cytokine expression. Similarly, transcriptomic analysis may represent an additional avenue for investigation in naturally infected animals either through whole tissue analysis, as has been done previously from fluke infected abattoir samples in sheep (Alvarez Rojas et al., 2015), or in specific cell phenotype responses such as in PBMCs *ex vivo* or following parasite-specific stimulation *in vitro* at different stages of infection.

6.2.1 Primary Exposure (Chapter 4)

In chapter 4, calves were sampled on a monthly basis. This may have reduced the amount of information obtained, although in reality the need for co-operation of the farmers and their staff in gathering and sampling the animals makes it unlikely visits of greater frequency would have been feasible. Nonetheless, this means there is some uncertainty as to exactly when infection had occurred; previous work validating the serum antibody ELISA demonstrated that sero-conversion occurs between 2-4 weeks of infection (Salimi-Bejestani et al., 2005b). Experimental studies have shown that the early stages of infection are associated with short-lived proliferative, mixed immune responses; typically 2-3 weeks post infection (Clery and Mulcahy, 1998; Oldham and Williams, 1985). These early immune responses may be of importance with respect to vaccine development, as there is evidence indicating that IgG2 antibody and ADCC responses have protective effects against the early migratory stages of infection in vaccinated cattle, goats and immunised rats (Golden et al., 2010; Mulcahy et al., 1998; Van Milligen et al., 1998; Zafra et al., 2013). We did not observe any statistically significant evidence of early proliferative or mixed cytokine responses following primary infection. However, this may have been due to the interval between samples being collected, resulting in such short-lived responses being missed, particularly since our data indicates that if positive proliferation and/or type-1 immune responses were present, this was more likely to be in the early stages of infection.

Future work investigating the immune responses of cattle to primary natural exposure could take one of two approaches; either more in depth analysis focusing on specific aspects of the immune response, or a more epidemiological approach relating immune responses to animal health and productivity.

In the case of the former, I would propose using smaller cohorts of 'sentinel' animals, specifically dairy bull calves, turned out to fluke positive pasture for the first time and observed over the course of the grazing season. The immune responses in these animals could either be characterised using the sort of methods we have used in this thesis or through the development of more integrative approaches such as multiple colour flow cytometry and/or transcriptomic analysis as previously discussed. The key difference between this study and that undertaken in chapter 4 would be more frequent (weekly) sampling in order to give a more detailed analysis of the early stages of infection, with culling and post-mortem of animals at the conclusion of the grazing season to establish presence of infection, burden, pathology etc. This approach would provide greater information concerning the infection itself and give greater access to sample material post mortem, including regional lymph nodes and hepatic tissue to allow the characterisation of the local immune responses. Bull calves rather than replacement dairy heifers would be used in this instance due to the high commercial and

genetic value of the latter. Use of sentinel animals would also provide considerable detail as to the specific nature of the immune responses of cattle to natural challenge with *Fasciola hepatica*, and potentially allow us to examine differences in protective versus non-protective immune responses to natural challenge through comparison to fluke burdens and pathology at post-mortem.

For the more epidemiological approach, increased sample size would be advisable to increase study power and ability to detect immunological changes of biological significance, including the presence of immune modulation and relationships with co-infecting pathogens. As a consequence, a more selective approach to both sample collection and immunological techniques would be required to allow larger sample numbers to be processed. For example, type-1 versus type-2 responses could be assessed through IgG isotype antibody responses (i.e. IgG2 vs IgG1) and/or whole blood stimulation assays rather than performing time-consuming leukocyte purification steps. Whilst this study would ultimately suffer from some of the same limitations as our current investigations, namely a lack of information about infection intensity and parasite burden, findings would ultimately be more directly applicable to the UK dairy industry and could provide valuable information on the impact of both *F. hepatica* itself and its associated immune responses on production and herd health.

6.2.2 Chronic Exposure (Chapter 5)

Chapter 5 as previously discussed suffered from a lack of overall power in some areas (Section 5.5). Further work using a larger number of animals from multiple dairy farms would be of benefit to the assessment of *F. hepatica* related effects on production, as this would allow evaluation at both the herd and individual level. The use of closantel to clear infection in dairy cattle over the dry period has previously been demonstrated to improve milk yield in the subsequent lactation (Charlier et al., 2012). Further investigation may also therefore help to establish whether this effect is also true with TCBZ, allowing the usefulness of a test and treat approach in controlling fasciolosis in dairy cattle at drying off to be assessed in terms of production and cost efficacy, which would clearly be of interest to UK dairy farmers. Increasing the power of this study may also allow for more detailed analysis of the effect of treatment on the host immune response. Further investigation in this area would be interesting, as it is possible that once active parasitic infection is cleared the observed immune-modulation may resolve potentially opening up the potential for vaccination in older previously infected cattle.

6.3 Multivariate Linear Mixed-Effects Model Analysis (Chapter 4)

Experimental studies are conducted in a controlled environment with defined infection protocols in terms of infectious dose, husbandry conditions etc. In Chapter 4, cattle were

sampled longitudinally over the period of a grazing season under normal farm management conditions. Consequently, a number of factors needed to be taken into account to allow meaningful data analysis. This was achieved using multivariable linear mixed-effects modelling. Whilst these statistical models are used routinely for epidemiological studies, to the best of our knowledge this is the first time such techniques have been used to analyse immunological responses to natural infection in livestock. The outputs from these models allowed us to properly evaluate the interactions between each immune parameter measured, and may represent a potentially useful template by which future studies may be designed to investigate immune responses in the natural context.

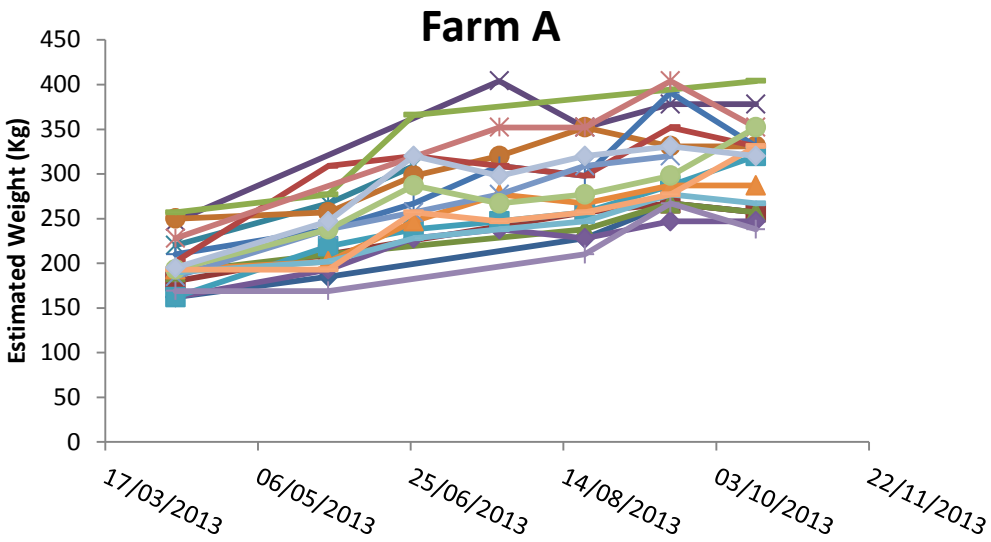
The longitudinal nature of our dataset required consideration in terms of the repeated sampling of individuals and the subsequent potential for auto-correlation, as seen with antibody PP values. Mixed effects linear regression models allowed both these issues to be considered appropriately; individual animals were modelled as a random effects variable to allow for inherent differences in individual animal responses, whilst an "AR-1 auto-correlation matrix" allowed us to account for the effect of temporal relatedness (Everitt and Hothorn, 2010).

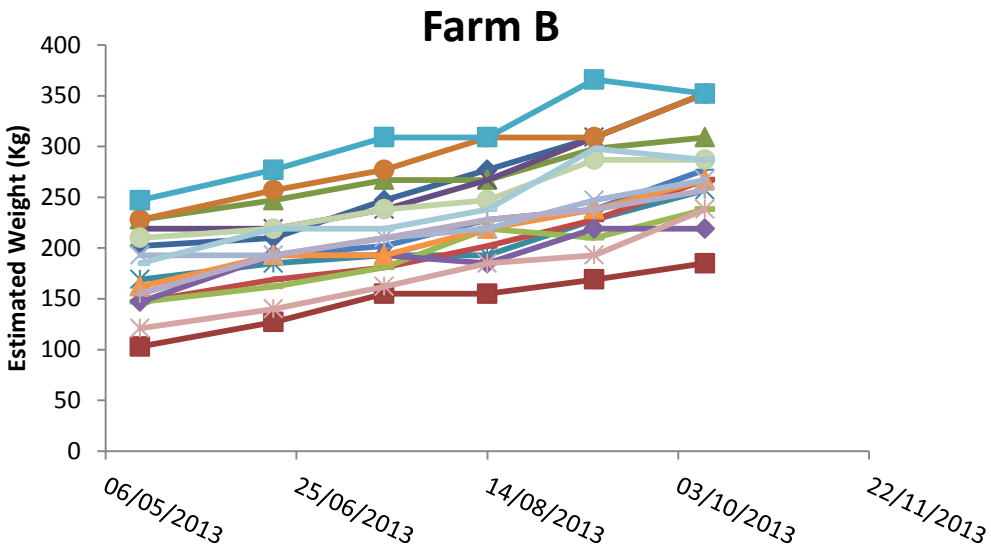
The multivariable component of the analysis also allowed for the simultaneous assessment of multiple explanatory variables to give a greater understanding of which parameters, immunological or otherwise, were the dominant features found to be associated with infection. Due to the assumed linear relationship between explanatory and response variables, it was necessary to check whether data transformation was needed ahead of model fit and perform Box-Cox transformation of the response variable as appropriate (Faraway, 2005). Formal stepwise selection was used as opposed to taking an intuitive approach, to allow for unbiased selection of significant explanatory variables.

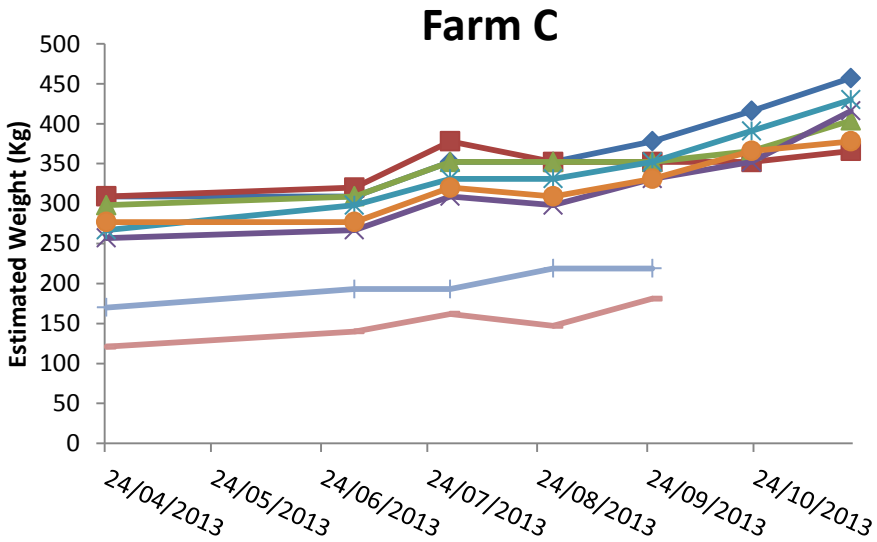
6.4 Conclusion and Summary

In conclusion, this thesis presents work which contributes to the current body of literature concerning the control of fasciolosis, demonstrating the efficacy of a simple but effective diagnostic tool for screening groups of cattle for infection, and through use of statistical and epidemiological modelling techniques provide evidence indicating that in response to infection with *F. hepatica* under natural field conditions, cattle exhibit a polarisation towards a non-proliferative type-2 immune response, with subsequent implications for both vaccination strategies and herd health and disease control. These findings also highlight the need for further research into areas of *F. hepatica* infection and immunology in cattle that could be of further benefit to the health, welfare and productivity of food producing animals and food security.

APPENDIX A: Growth Charts for Individual Animals from Primary Exposure Study (Chapter 2)







APPENDIX B: Supplementary Material for Assay Kit Protocols and Reagent Recipes (Chapter 2)

B.1 Dilution protocol of BSA standards for Bradford assay

Taken from 'protocol B, microplate procedure', Thermo Scientific instruction manual (Cat No. 23236)

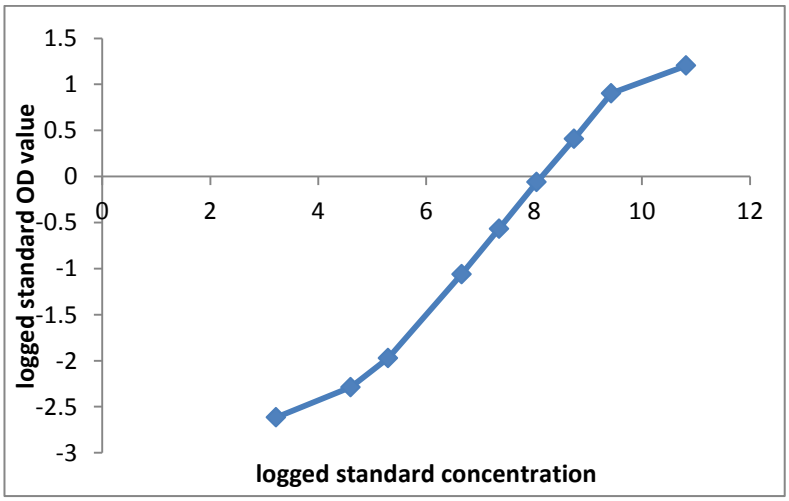
Standard ID	Volume of dH_2O diluents (µl)	Volume and BSA source (µl)	Final BSA Concentration (µg/ml)
A	3555	45 (stock)	25
B	6435	65 (stock)	20
C	3970	30 (stock)	15
D	3000	3000 (vial D)	10
E	2500	3000 (vial B)	5
F	1700	1700 (vial E)	2.5
G	4000	0	0

B.2 **Bovine IFN-γ ELISA kit** (AbD Serotec, Cat no.MCA5638KZZ)

Standard curve dilutions, taken from 'Bovine IFN-g ELISA Kit Insert'

Standard ID	Concentration (ng/ml)	Composition
A	50	50µl standard stock + 350µl wash buffer
B	12.5	150µl standard A + 450µl wash buffer
C	6.25	250µl standard B + 250µl wash buffer
D	3.13	250µl standard C + 250µl wash buffer
E	1.56	250µl standard D + 250µl wash buffer
F	0.78	250µl standard E + 250µl wash buffer
G	0.2	150µl standard F + 450µl wash buffer
H	0.1	250µl standard G + 250µl wash buffer
I	0.025	100µl standard H + 300µl wash buffer

Example log-transformed standard curve (14/11/13):



Example point-to-point regression values (14/11/13)

Standard range (ng/ml)	point to point regression values for log-transformed values	
	x-intercept	slope value
50 - 12.5	-1.15041	0.217683
12.5 - 6.25	-5.83877	0.714675
6.25 - 3.13	-5.5228	0.678524
3.13 - 1.56	-5.90687	0.726242
1.56 - 0.78	-5.79754	0.711372
0.78 - 0.2	-5.52049	0.669768
0.2 - 0.1	-4.38618	0.455679
0.1 - 0.025	-3.37578	0.236275

B.3 Bovine IL-4 ELISA kit (AbD Serotec , Cat no.MCA5892KZZ)

Standard curve dilutions, taken from 'Bovine IL-4 ELISA Kit Insert'

Standard ID	Concentration (pg/ml)	Composition
A	2000	Reconstituted standard in 500µl in dH_20
B	1000	250µl standard A + 250µl wash buffer
C	500	250µl standard B + 250µl wash buffer
D	100	250µl standard C + 800µl wash buffer
E	50	250µl standard D + 250µl wash buffer
F	10	250µl standard E + 800µl wash buffer
G	2.5	250µl standard F + 750µl wash buffer
H	0	500µl wash buffer

B.4 0.1M Carbonate coating buffer

Add and dissolve 0.159g Na_2CO_3 (anhydrous) & 0.292g $NaHCO_3$ to 100ml dH_2O. Adjust pH to 9.6 by adding 10% HCl as required. Refresh weekly.

B.5 Phosphate Buffered Saline with Tween-20 and associated preparations

Initially prepare a 5x PBS stock solution:

Add and dissolve 90g NaCl, 18.5g Na_2HPO_4 & 5.375g KH_2PO_4 in 2.5L dH_2O.

PBS 0.05% Tween wash buffer:

Add and dissolve 500ml 5x stock solution & 1.25ml of Tween-20 in 2L dH_2O.

2% Marvel blocking buffer:

Add and dissolve 2g of Marvel (Chivers Ireland Ltd, Dublin Ireland) in 100ml PBS w/0.05% Tween-20 wash buffer. Refresh weekly.

0.1% Caesin blocking buffer:

Add and dissolve 0.1g sodium casein salt in 100ml PBS w/0.05% Tween-20 wash buffer. Refresh weekly.

B.6 Tricine Buffered Saline

As described as 'Solution C' in the Axis-Shield application sheet C08. Though not essential this layer is designed to prevent PBMC adherence to the side of the falcon tubes, thereby increasing yield. Preparation as follows:

Add and dissolve 1.79g tricine in 100ml dH_2O to make a 100mM tricine solution.

Add and dissolve 4.25g NaCl in 250ml dH_2O.

Add 100ml of 100mM tricine solution to 250ml NaCl solution and adjust the pH of the solution to 7.4 using 1M NaOH solution. Make the final volume up to 500ml using dH_2O.

Finally, in a tissue culture hood filter sterilise (VWR, Radnor USA) the solution prior to use with PBMCs.

B.7 TGF-β1 carbonate coating buffer

As described in Promega Technical Bulletin TB196:

Prepare a solution of concentration 0.025M sodium carbonate and 0.025M sodium bicarbonate by adding and dissolving 0.265g Na_2CO_3 and 0.21g $NaHCO_3$ in 100ml dH_2O. Adjust pH to 9.7 by adding 1N HCl or 1N NaOH as required. Refresh weekly.

B.8 TGF-β1 TBST wash buffer

As described in Promega Technical Bulletin TB196:

Prepare a solution consisting of 20mM Tris-HCl, 150mM NaCl and 0.05% (v/v) Tween® 20; Add and dissolve 7.85g Tris-HCl, 21.75g NaCl and 1.25ml Tween-20 in 2.5L dH_2O. Adjust pH to 7.6 by adding 1N HCl or 1N NaOH as required. Refresh weekly.

B.9 2% Acetic acid with 1% gentian violet solution

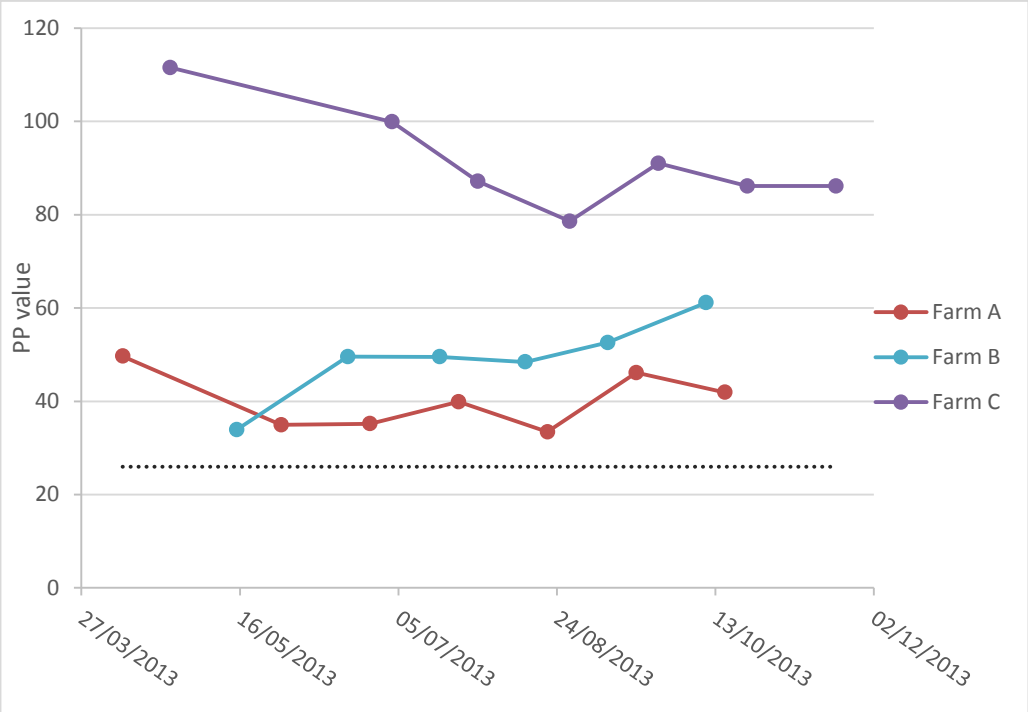
Add and mix 2ml glacial acetic acid and 1ml of a 1% aqueous solution of gentian violet to 100ml dH_2O.

APPENDIX C: Summarised Fluke Egg Count Data for Farms B.1 to B.22 (Chapter 3)

Includes group size, individual egg counts and fitted Negative Binomial Distribution (NBD) parameters with NBD-predicted composite egg counts.

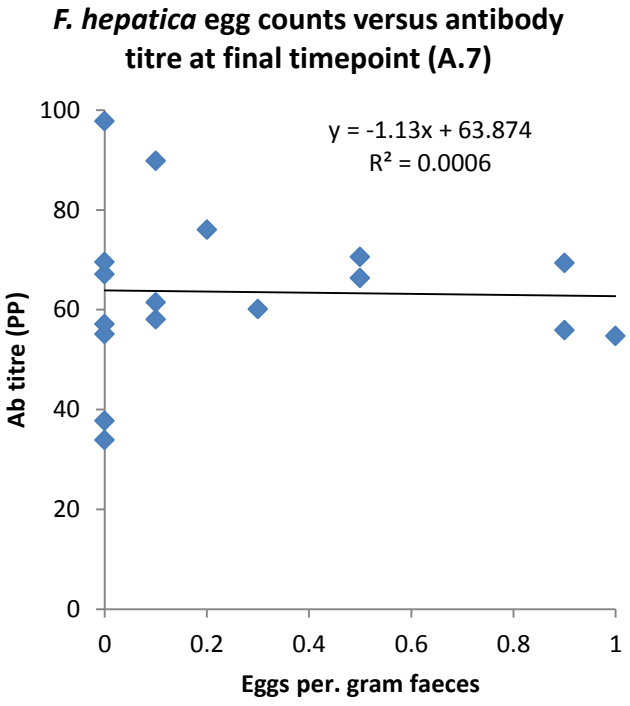
ID	group size (n)	Prevalence (%)	Sum of individual counts (50g)	NBD fit parameters			NBD-predicted composite counts (50g)	
				mean (μ)	k-value	X^2 p-value	Mean	SD (σ)
B.1	26	42.308	175 25	2.077	0.237	0.998	104.562	71.820
B.2	24	8.333	0 10	0.083	100	0.925	4.114	4.484
B.3	25	60	150 60	2.320	0.555	0.827	116.632	55.628
B.4	28	64.286	130 130	2.071	0.825	0.319	104.308	43.134
B.5	25	96	220 315	4.720	3.121	0.759	235.640	54.633
B.6	25	68	110 65	1.640	1.352	0.852	82.040	29.668
B.7	25	20	10 35	0.360	0.252	0.967	17.799	14.456
B.8	25	72	110 155	2.920	0.891	0.834	145.374	56.294
B.9	24	33.333	25 120	1.625	0.193	0.992	81.953	62.500
B.10	20	35	10 45	0.550	0.811	0.614	27.479	15.047
B.11	20	40	15 25	0.400	100	0.467	19.792	9.941
B.12	20	35	15 110	1.250	0.240	0.350	62.687	44.171
B.13	20	85	50 95	1.450	8.364	0.667	72.590	20.838
B.14	20	35	50 45	0.950	0.307	0.754	47.253	31.142
B.15	20	40	45 35	0.800	0.675	0.687	40	20.913
B.16	20	70	105 130	2.350	0.867	0.676	116.484	46.511
B.17	33	3.030	0 0 5	0.030	100	1.000	1.524	2.750
B.18	20	15	15 0	0.150	100	0.349211	7.499	6.141
B.19	20	5	0 25	0.250	0.020	1	12.49	28.793
B.20	20	5	10 0	0.100	0.044	1	4.991	9.042
B.21	20	10	0 10	0.100	100	0.574	4.951	4.997
B.22	20	70	70 130	2.000	1.097	0.707	100.568	38.123

APPENDIX D: Supplementary BMT, Fluke and Nematode Egg Count Data from Farms A, B and C (Chapter 4)



***F. hepatica* Bulk Milk Tank antibody ELISA PP values for farms A, B and C.** Values recorded from April to November 2013 (study period). Dashed line denotes the positive cut-off value (≥27PP) defined for BMT samples (Salimi-Bejestani et al., 2005a).

	Fluke eggs	
Tag No	*F. hepatica*	*Paramphistome*
2591	0.3	0.4
2613	0.1	0
2622	0.9	0.1
2632	0.2	0.1
2642	1	0
2644	0.9	0.8
2651	0.5	0.7
2658	0	0
2663	0	0
2664	0	0.1
2673	0	0
2679	0	0
2685	0.1	0
2686	0.1	0.2
2687	0.5	0
2689	0	0
2720	0	0
Mean	0.270588	0.141176
SD	0.356659	0.252633



Fluke egg counts Farm "A", final time point (A.7). All other composite and individual fluke egg sedimentations from other farms and time points were negative.

Nematode egg counts for each farm as measured over the course of the study period. †Denotes treatment of cohort group with ivermectin *Denotes *Nematodirus spp.* infections. ₸Denotes mixed trichostrongyle and *Nematodirus spp.* infections.

Farm A	Month of Sampling					
Tag No.	**A.2†**	**A.3**	**A.4**	**A.5†**	**A.6**	**A.7†**
2591	0	NA	NA	0	0	0
2613	0	0	0	0	0	0
2622	NA	NA	50	0	0	0
2632	NA	NA	50	0	0	0
2642	0	0	NA	NA	NA	0
2644	0	0	0	0	0	0
2651	0	0	0	0	0	0
2658	0	0	0	0	0	0
2663	0	NA	NA	0	0	0
2664	0	0	0	0	0	0
2666	0	0	0	0	0	NA
2667	0	0	NA	NA	NA	NA
2673	0	NA	NA	0	0	0
2679	0	0	0	0	0	0
2685	0	0	0	0	0	0
2686	0	0	0	0	0	0
2687	0	0	0	0	0	0
2689	0	0	0	0	0	0
2720	0	NA	NA	0	0	0

Farm B	Month of Sampling				
Tag No.	**B.2**	**B.3**	**B.4**	**B.5†**	**B.6**
100986	0	0	0	0	0
100993	0	0	50	0	0
200987	0	0	50	50	150*
300988	0	0	100	150	0
300995	0	0	50	50*	100*
400982	0	0	100	100	0
400989	0	0	0	0	0
400996	0	0	0	100	0
500990	0	0	0	100	0
500997	0	0	0	100	0
600977	0	0	50	100	0
600984	0	0	0	50	0
600991	0	0	0	50	0
600998	0	0	100	550₸	0
700985	0	0	0	50	0
700992	0	0	0	100	0
700999	0	0	50	150	0

Farm C	Month of Sampling					
Tag No	**C.2**	**C.3**	**C.4**	**C.5**	**C.6†**	**C.7**
465	0	0	0	0	100	0
466	0	200	0	0	500	0
469	0	50	0	0	150	0
475	0	50	0	0	0	0
476	0	0	0	0	0	0
481	0	0	0	0	0	0
494	0	0	0	0	NA	NA
503	0	0	0	0	NA	NA

References

Abbott, J.R., Palmer, G.H., Kegerreis, K.A., Hetrick, P.F., Howard, C.J., Hope, J.C., Brown, W.C., 2005. Rapid and long-term disappearance of CD4+ T lymphocyte responses specific for Anaplasma marginale major surface protein-2 (MSP2) in MSP2 vaccinates following challenge with live A. marginale. J Immunol 174, 6702-6715.

Adams, P.N., Aldridge, A., Vukman, K.V., Donnelly, S., O'Neill, S.M., 2014. Fasciola hepatica tegumental antigens indirectly induce an M2 macrophage-like phenotype in vivo. Parasite immunology 36, 531-539.

Ai, L., Li, C., Elsheikha, H.M., Hong, S.J., Chen, J.X., Chen, S.H., Li, X., Cai, X.Q., Chen, M.X., Zhu, X.Q., 2010. Rapid identification and differentiation of Fasciola hepatica and Fasciola gigantica by a loop-mediated isothermal amplification (LAMP) assay. Veterinary parasitology 174, 228-233.

Aitken, M.M., Hughes, D.L., Jones, P.W., Hall, G.A., Smith, G.S., 1979. Immunological responses of fluke-infected and fluke-free cattle to Salmonella dublin and other antigens. Research in veterinary science 27, 306-312.

Alba, A., Vazquez, A.A., Hernandez, H., Sanchez, J., Marcet, R., Figueredo, M., Sarracent, J., Fraga, J., 2015. A multiplex PCR for the detection of Fasciola hepatica in the intermediate snail host Galba cubensis. Veterinary parasitology 211, 195-200.

Alexandratos, N., Bruinsma, J. 2012. World agriculture towards 2030/2050: the 2012 revision (Rome, FAO).

Almeida, M.S., Torloni, H., Lee-Ho, P., Vilar, M.M., Thaumaturgo, N., Simpson, A.J., Tendler, M., 2003. Vaccination against Fasciola hepatica infection using a Schistosoma mansoni defined recombinant antigen, Sm14. Parasite immunology 25, 135-137.

Alvarez Rojas, C.A., Ansell, B.R., Hall, R.S., Gasser, R.B., Young, N.D., Jex, A.R., Scheerlinck, J.P., 2015. Transcriptional analysis identifies key genes involved in metabolism, fibrosis/tissue repair and the immune response against Fasciola hepatica in sheep liver. Parasites & vectors 8, 124.

Anderson, N., Luong, T.T., Vo, N.G., Bui, K.L., Smooker, P.M., Spithill, T.W., 1999. The sensitivity and specificity of two methods for detecting Fasciola infections in cattle. Veterinary Parasitology 83, 15-24.

Anderson, P.H., Berrett, S., Brush, P.J., Hebert, C.N., Parfitt, J.W., Patterson, D.S., 1977. Biochemical indicators of liver injury in calves with experimental fascioliasis. Vet Rec 100, 43-45.

Anderson, R.M., Gordon, D.M., 1982. Processes influencing the distribution of parasite numbers within host populations with special emphasis on parasite-induced host mortalities. Parasitology 85 (Pt 2), 373-398.

Anderson, R.M., May, R.M., 1991. Infectious diseases of humans: dynamics and control. Oxford University Press, Oxford.

Andrews, S.J., 1999. The Life Cycle of *Fasciola hepatica*, In: Dalton, J.P. (Ed.) Fasciolosis. CABI publishing.

ANON 2007. AHVLA Standard Operating Procedure, SOP PA026, Fluke egg demonstration by sedimentation.

Anon., 1986. Manual of Veterinary Parasitological Techniques, 3rd Edition. MAFF, London: HMSO.

Anon. 2007. Fluke egg demonstration by sedimentation. In SOP PA026 (AHVLA Standard Operating Procedure).

Arias, M.S., Pineiro, P., Sanchez-Andrade, R., Suarez, J.L., Hillyer, G.V., Diez-Banos, P., Paz-Silva, A., Morrondo, P., 2013. Relationship between exposure to Fasciola hepatica in roe deer (Capreolus capreolus) and cattle extensively reared in an endemic area. Research in veterinary science 95, 1031-1035.

Arjona, R., Riancho, J.A., Aguado, J.M., Salesa, R., Gonzalez-Macias, J., 1995. Fascioliasis in developed countries: a review of classic and aberrant forms of the disease. Medicine 74, 13-23.

Armour, J., Dargie, J.D., 1974. Immunity to Fasciola hepatica in the rat. Experimental parasitology 35, 381-388.

Bagadi, H.O., Sewell, M.M., 1973. An epidemiological survey of infectious necrotic hepatitis (Black Disease) of sheep in Southern Scotland. Research in veterinary science 15, 49-53.

Baker, C. 2015. Dairy industry in the UK: statistics. In Social & General Statistics Section, library, H.o.C., ed.

Bargues, M.D., Gonzalez, L.C., Artigas, P., Mas-Coma, S., 2011. A new baseline for fascioliasis in Venezuela: lymnaeid vectors ascertained by DNA sequencing and analysis of their relationships with human and animal infection. Parasites & vectors 4, 200.

Bargues, M.D., Mas-Coma, S., 2005. Reviewing lymnaeid vectors of fascioliasis by ribosomal DNA sequence analyses. Journal of helminthology 79, 257-267.

Bartley, D.J., Jackson, F., Jackson, E., Sargison, N., 2004. Characterisation of two triple resistant field isolates of Teladorsagia from Scottish lowland sheep farms. Veterinary parasitology 123, 189-199.

Bassetto, C.C., Silva, M.R., Newlands, G.F., Smith, W.D., Ratti Junior, J., Martins, C.L., Amarante, A.F., 2014. Vaccination of grazing calves with antigens from the intestinal membranes of Haemonchus contortus: effects against natural challenge with Haemonchus placei and Haemonchus similis. International journal for parasitology 44, 697-702.

Behm, C.A., Sangster, N.C., 1999. Pathology, Pathophysiology and Clinical Aspects, In: Dalton, J.P. (Ed.) Fasciolosis. CABI publishing.

Bennet, R., Ijpelaar, J. 2003. Economic assessment of livestock diseases in Great Britain. Final report to Defra, Defra, ed. (University of Reading).

Bennett, C.E., Threadgold, L.T., 1975. Fasciola hepatica: development of tegument during migration in mouse. Experimental parasitology 38, 38-55.

Berasain, P., Carmona, C., Frangione, B., Dalton, J.P., Goni, F., 2000. Fasciola hepatica: parasite-secreted proteinases degrade all human IgG subclasses: determination of the specific cleavage sites and identification of the immunoglobulin fragments produced. Experimental parasitology 94, 99-110.

Berasain, P., Goni, F., McGonigle, S., Dowd, A., Dalton, J.P., Frangione, B., Carmona, C., 1997. Proteinases secreted by Fasciola hepatica degrade extracellular matrix and basement membrane components. The Journal of parasitology 83, 1-5.

Boray, J.C., 1969. Experimental fascioliasis in Australia. Advances in parasitology 7, 95-210.

Boray, J.C., Crowfoot, P.D., Strong, M.B., Allison, J.R., Schellenbaum, M., Von Orelli, M., Sarasin, G., 1983. Treatment of immature and mature Fasciola hepatica infections in sheep with triclabendazole. Vet Rec 113, 315-317.

Bosco, A., Rinaldi, L., Musella, V., Amadesi, A., Cringoli, G., 2015. Outbreak of acute fasciolosis in sheep farms in a Mediterranean area arising as a possible consequence of climate change. Geospatial health 9, 319-324.

Bossaert, K., Farnir, F., Leclipteux, T., Protz, M., Lonneux, J.F., Losson, B., 2000a. Humoral immune response in calves to single-dose, trickle and challenge infections with Fasciola hepatica. Veterinary parasitology 87, 103-123.

Bossaert, K., Jacquinet, E., Saunders, J., Farnir, F., Losson, B., 2000b. Cell-mediated immune response in calves to single-dose, trickle, and challenge infections with Fasciola hepatica. Veterinary parasitology 88, 17-34.

Brady, M.T., O'Neill, S.M., Dalton, J.P., Mills, K.H., 1999. Fasciola hepatica suppresses a protective Th1 response against Bordetella pertussis. Infection and immunity 67, 5372-5378.

Brockwell, Y.M., Elliott, T.P., Anderson, G.R., Stanton, R., Spithill, T.W., Sangster, N.C., 2014. Confirmation of Fasciola hepatica resistant to triclabendazole in naturally infected Australian beef and dairy cattle. International journal for parasitology. Drugs and drug resistance 4, 48-54.

Brown, W.C., Davis, W.C., Dobbelaere, D.A., Rice-Ficht, A.C., 1994. CD4+ T-cell clones obtained from cattle chronically infected with Fasciola hepatica and specific for adult worm antigen express both unrestricted and Th2 cytokine profiles. Infection and immunity 62, 818-827.

Caban-Hernandez, K., Gaudier, J.F., Ruiz-Jimenez, C., Espino, A.M., 2014. Development of two antibody detection enzyme-linked immunosorbent assays for serodiagnosis of human chronic fascioliasis. Journal of clinical microbiology 52, 766-772.

Caminade, C., van Dijk, J., Baylis, M., Williams, D., 2015. Modelling recent and future climatic suitability for fasciolosis in Europe. Geospatial health 9, 301-308.

Campbell, N.J., Gregg, P., Kelly, J.D., Dineen, J.K., 1978. Failure to induce homologous immunity to Fasciola hepatica in sheep vaccinated with irradiated metacercariae. Veterinary parasitology 4, 143-152.

Cancela, M., Acosta, D., Rinaldi, G., Silva, E., Duran, R., Roche, L., Zaha, A., Carmona, C., Tort, J.F., 2008. A distinctive repertoire of cathepsins is expressed by juvenile invasive Fasciola hepatica. Biochimie 90, 1461-1475.

Carmona, C., Dowd, A.J., Smith, A.M., Dalton, J.P., 1993. Cathepsin L proteinase secreted by Fasciola hepatica in vitro prevents antibody-mediated eosinophil attachment to newly excysted juveniles. Molecular and biochemical parasitology 62, 9-17.

Castro, E., Freyre, A., Hernandez, Z., 2000. Serological responses of cattle after treatment and during natural re-infection with Fasciola hepatica, as measured with a dot-ELISA system. Vet Parasitol 90, 201-208.

CDI 2013. Breed Performance Statistics, Richardson, H., ed.

Ceballos, L., Moreno, L., Alvarez, L., Shaw, L., Fairweather, I., Lanusse, C., 2010. Unchanged triclabendazole kinetics after co-administration with ivermectin and methimazole: failure of its therapeutic activity against triclabendazole-resistant liver flukes. BMC veterinary research 6, 8.
Cervi, L., Rossi, G., Masih, D.T., 1999. Potential role for excretory-secretory forms of glutathione-S-transferase (GST) in Fasciola hepatica. Parasitology 119, 627-633.
Chapman, C.B., Mitchell, G.F., 1982. Proteolytic cleavage of immunoglobulin by enzymes released by Fasciola hepatica. Veterinary parasitology 11, 165-178.
Charlier, J., De Meulemeester, L., Claerebout, E., Williams, D., Vercruysse, J., 2008a. Qualitative and quantitative evaluation of coprological and serological techniques for the diagnosis of fasciolosis in cattle. Veterinary parasitology 153, 44-51.
Charlier, J., Duchateau, L., Claerebout, E., Williams, D., Vercruysse, J., 2007. Associations between anti-Fasciola hepatica antibody levels in bulk-tank milk samples and production parameters in dairy herds. Preventive veterinary medicine 78, 57-66.
Charlier, J., Hostens, M., Jacobs, J., Van Ranst, B., Duchateau, L., Vercruysse, J., 2012. Integrating fasciolosis control in the dry cow management: the effect of closantel treatment on milk production. PloS one 7, e43216.
Charlier, J., Vercruysse, J., Morgan, E., van Dijk, J., Williams, D.J., 2014. Recent advances in the diagnosis, impact on production and prediction of Fasciola hepatica in cattle. Parasitology 141, 326-335.
Chiodini, R.J., Davis, W.C., 1992. The cellular immunology of bovine paratuberculosis: the predominant response is mediated by cytotoxic gamma/delta T lymphocytes which prevent CD4+ activity. Microbial pathogenesis 13, 447-463.
Chitko-McKown, C.G., Ruef, B.J., Rice-Ficht, A.C., Brown, W.C., 1995. Interleukin-10 downregulates proliferation and expression of interleukin-2 receptor p55 chain and interferon-gamma, but not interleukin-2 or interleukin-4, by parasite-specific helper T cell clones obtained from cattle chronically infected with Babesia bovis or Fasciola hepatica. Journal of interferon & cytokine research. 15, 915-922.
Claridge, J., Diggle, P., McCann, C.M., Mulcahy, G., Flynn, R., McNair, J., Strain, S., Welsh, M., Baylis, M., Williams, D.J., 2012. Fasciola hepatica is associated with the failure to detect bovine tuberculosis in dairy cattle. Nature communications 3, 853.
Clery, D., O'Mahony, P., Mulcahy, G., 1995. A novel method for the administration of metacercariae of Fasciola hepatica to adult cattle. Research in veterinary science 58, 290-291.
Clery, D., Torgerson, P., Mulcahy, G., 1996. Immune responses of chronically infected adult cattle to Fasciola hepatica. Veterinary parasitology 62, 71-82.
Clery, D.G., Mulcahy, G., 1998. Lymphocyte and cytokine responses of young cattle during primary infection with Fasciola hepatica. Research in veterinary science 65, 169-171.
Clevers, H., MacHugh, N.D., Bensaid, A., Dunlap, S., Baldwin, C.L., Kaushal, A., Iams, K., Howard, C.J., Morrison, W.I., 1990. Identification of a bovine surface antigen uniquely expressed on CD4-CD8- T cell receptor gamma/delta+ T lymphocytes. European journal of immunology 20, 809-817.
Conceicao, M.A., Durao, R.M., Costa, I.H., da Costa, J.M., 2002. Evaluation of a simple sedimentation method (modified McMaster) for diagnosis of bovine fascioliosis. Veterinary parasitology 105, 337-343.
Corba, J., Armour, J., Roberts, R.J., Urquhart, G.M., 1971. Transfer of immunity to Fasciola hepatica infection by lymphoid cells. Research in veterinary science 12, 292-295.
Coussens, P.M., Sipkovsky, S., Murphy, B., Roussey, J., Colvin, C.J., 2012. Regulatory T cells in cattle and their potential role in bovine paratuberculosis. Comparative immunology, microbiology and infectious diseases 35, 233-239.
COWS 2013. Sustainable worm control strategies for cattle. In Control of Worms Sustainably (Agricultural and Horticultural Development Board).
Creaney, J., Spithill, T.W., Thompson, C.M., Wilson, L.R., Sandeman, R.M., Parsons, J.C., 1995. Attempted immunisation of sheep against Fasciola hepatica using gamma-irradiated metacercariae. International journal for parasitology 25, 853-856.
Cringoli, G., Rinaldi, L., Maurelli, M.P., Utzinger, J., 2010. FLOTAC: new multivalent techniques for qualitative and quantitative copromicroscopic diagnosis of parasites in animals and humans. Nature protocols 5, 503-515.
Cuervo, P.F., Cataldo, S.D., Fantozzi, M.C., Deis, E., Isenrath, G.D., Viberti, G., Artigas, P., Peixoto, R., Valero, M.A., Sierra, R.M., Mas-Coma, S., 2015. Liver fluke (Fasciola hepatica) naturally infecting introduced European brown hare (Lepus europaeus) in northern Patagonia: phenotype,

prevalence and potential risk. Acta parasitologica / Witold Stefanski Institute of Parasitology, Warszawa, Poland 60, 536-543.
Cui, L., Mharakurwa, S., Ndiaye, D., Rathod, P.K., Rosenthal, P.J., 2015. Antimalarial Drug Resistance: Literature Review and Activities and Findings of the ICEMR Network. The American journal of tropical medicine and hygiene 93, 57-68.
Cwiklinski, K., Dalton, J.P., Dufresne, P.J., La Course, J., Williams, D.J., Hodgkinson, J., Paterson, S., 2015. The Fasciola hepatica genome: gene duplication and polymorphism reveals adaptation to the host environment and the capacity for rapid evolution. Genome biology 16, 71.
Dalton, J.P., Robinson, M.W., Mulcahy, G., O'Neill, S.M., Donnelly, S., 2013. Immunomodulatory molecules of Fasciola hepatica: candidates for both vaccine and immunotherapeutic development. Veterinary parasitology 195, 272-285.
Daniel, R., van Dijk, J., Jenkins, T., Akca, A., Mearns, R., Williams, D.J., 2012. Composite faecal egg count reduction test to detect resistance to triclabendazole in Fasciola hepatica. Vet Rec 171, 153, 151-155.
Dargie, J.D., 1987. The impact on production and mechanisms of pathogenesis of trematode infections in cattle and sheep. International journal for parasitology 17, 453-463.
Davies, C., Goose, J., 1981. Killing of newly excysted juveniles of Fasciola hepatica in sensitized rats. Parasite immunology 3, 81-96.
de Almeida, D.E., Colvin, C.J., Coussens, P.M., 2008. Antigen-specific regulatory T cells in bovine paratuberculosis. Veterinary immunology and immunopathology 125, 234-245.
DEFRA 2014. The Strategy for achieving Officially Bovine Tuberculosis Free status for England, &, D.f.E.F., ed.
Dixon, K.E., 1964. THE RELATIVE SUITABILITY OF SHEEP AND CATTLE AS HOSTS FOR THE LIVER FLUKE, FASCIOLA HEPATICA L. Journal of helminthology 38, 203-212.
Dixon, K.E., 1965. THE STRUCTURE AND HISTOCHEMISTRY OF THE CYST WALL OF THE METACERCARIA OF FASCIOLA HEPATICA L. Parasitology 55, 215-226.
Dixon, K.E., 1966. The physiology of excystment of the metacercaria of Fasciola hepatica L. Parasitology 56, 431-456.
Donnelly, S., O'Neill, S.M., Sekiya, M., Mulcahy, G., Dalton, J.P., 2005. Thioredoxin peroxidase secreted by Fasciola hepatica induces the alternative activation of macrophages. Infection and immunity 73, 166-173.
Donnelly, S., O'Neill, S.M., Stack, C.M., Robinson, M.W., Turnbull, L., Whitchurch, C., Dalton, J.P., 2010. Helminth cysteine proteases inhibit TRIF-dependent activation of macrophages via degradation of TLR3. The Journal of biological chemistry 285, 3383-3392.
Donnelly, S., Stack, C.M., O'Neill, S.M., Sayed, A.A., Williams, D.L., Dalton, J.P., 2008. Helminth 2-Cys peroxiredoxin drives Th2 responses through a mechanism involving alternatively activated macrophages. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 22, 4022-4032.
Dowd, A.J., McGonigle, S., Dalton, J.P., 1995. Fasciola hepatica cathepsin L proteinase cleaves fibrinogen and produces a novel type of fibrin clot. European journal of biochemistry / FEBS 232, 241-246.
Dowling, D.J., Hamilton, C.M., Donnelly, S., La Course, J., Brophy, P.M., Dalton, J., O'Neill, S.M., 2010. Major secretory antigens of the helminth Fasciola hepatica activate a suppressive dendritic cell phenotype that attenuates Th17 cells but fails to activate Th2 immune responses. Infection and immunity 78, 793-801.
Duff, J.P., Maxwell, A.J., Claxton, J.R., 1999. Chronic and fatal fascioliasis in llamas in the UK. Vet Rec 145, 315-316.
Duffus, W.P., Franks, D., 1980. In vitro effect of immune serum and bovine granulocytes on juvenile Fasciola hepatica. Clinical and experimental immunology 41, 430-440.
Duffus, W.P., Thorne, K., Oliver, R., 1980. Killing of juvenile Fasciola hepatica by purified bovine eosinophil proteins. Clinical and experimental immunology 40, 336-344.
Duscher, R., Duscher, G., Hofer, J., Tichy, A., Prosl, H., Joachim, A., 2011. Fasciola hepatica - monitoring the milky way? The use of tank milk for liver fluke monitoring in dairy herds as base for treatment strategies. Veterinary parasitology 178, 273-278.
Dyce, K.M., Sack, W.O., Wensing, C.J.G., 2002. Textbook of veterinary anatomy. Philadelphia, PA : Saunders, 2002. 3rd ed.
EBLEX 2011. Stock Briefing.
EBLEX 2015. Stock Briefing: Improving the control of liver fluke in UK cattle and sheep.
Estes, D.M., Closser, N.M., Allen, G.K., 1994. IFN-gamma stimulates IgG2 production from bovine B cells costimulated with anti-mu and mitogen. Cellular immunology 154, 287-295.

Everitt, B., Hothorn, T., 2010. A handbook of statistical analyses using R. [electronic book]. Boca Raton FL ; CRC/Chapman & Hall, 2010. 2nd ed.
Fairweather, I., 2011. Reducing the future threat from (liver) fluke: realistic prospect or quixotic fantasy? Veterinary parasitology 180, 133-143.
Fairweather, I., Boray, J.C., 1999. Fasciolicides: efficacy, actions, resistance and its management. Vet J 158, 81-112.
Fairweather, I., Threadgold, L.T., Hanna, R.E., 1999. Development of *Fasciola hepatica* in the Mammalian Host, In: Dalton, J.P. (Ed.) Fasciolosis. CABI publishing.
Falcon, C., Carranza, F., Martinez, F.F., Knubel, C.P., Masih, D.T., Motran, C.C., Cervi, L., 2010. Excretory-secretory products (ESP) from Fasciola hepatica induce tolerogenic properties in myeloid dendritic cells. Veterinary immunology and immunopathology 137, 36-46.
Falcon, C.R., Masih, D., Gatti, G., Sanchez, M.C., Motran, C.C., Cervi, L., 2014. Fasciola hepatica Kunitz type molecule decreases dendritic cell activation and their ability to induce inflammatory responses. PloS one 9, e114505.
FAO 2011. Global food losses and waste - Extent, causes and prevention (Rome).
Faraway, J.J., 2005. Linear models with R. Boca Raton ; Chapman & Hall/CRC.
Figueroa-Santiago, O., Espino, A.M., 2014. Fasciola hepatica fatty acid binding protein induces the alternative activation of human macrophages. Infection and immunity 82, 5005-5012.
Fitzpatrick, J.L., 2013. Global food security: the impact of veterinary parasites and parasitologists. Veterinary parasitology 195, 233-248.
Flynn, R.J., Irwin, J.A., Olivier, M., Sekiya, M., Dalton, J.P., Mulcahy, G., 2007a. Alternative activation of ruminant macrophages by Fasciola hepatica. Veterinary immunology and immunopathology 120, 31-40.
Flynn, R.J., Mannion, C., Golden, O., Hacariz, O., Mulcahy, G., 2007b. Experimental Fasciola hepatica infection alters responses to tests used for diagnosis of bovine tuberculosis. Infection and immunity 75, 1373-1381.
Flynn, R.J., Mulcahy, G., 2008a. Possible role for Toll-like receptors in interaction of Fasciola hepatica excretory/secretory products with bovine macrophages. Infection and immunity 76, 678-684.
Flynn, R.J., Mulcahy, G., 2008b. The roles of IL-10 and TGF-beta in controlling IL-4 and IFN-gamma production during experimental Fasciola hepatica infection. International journal for parasitology 38, 1673-1680.
Flynn, R.J., Mulcahy, G., Elsheikha, H.M., 2010. Coordinating innate and adaptive immunity in Fasciola hepatica infection: implications for control. Veterinary parasitology 169, 235-240.
Flynn, R.J., Mulcahy, G., Welsh, M., Cassidy, J.P., Corbett, D., Milligan, C., Andersen, P., Strain, S., McNair, J., 2009. Co-Infection of cattle with Fasciola hepatica and Mycobacterium bovis- immunological consequences. Transboundary and emerging diseases 56, 269-274.
Fox, N.J., White, P.C., McClean, C.J., Marion, G., Evans, A., Hutchings, M.R., 2011. Predicting impacts of climate change on Fasciola hepatica risk. PloS one 6, e16126.
Fraser, D., Duncan, I.J., Edwards, S.A., Grandin, T., Gregory, N.G., Guyonnet, V., Hemsworth, P.H., Huertas, S.M., Huzzey, J.M., Mellor, D.J., Mench, J.A., Spinka, M., Whay, H.R., 2013. General Principles for the welfare of animals in production systems: the underlying science and its application. Vet J 198, 19-27.
Freeman, A.R., Lynn, D.J., Murray, C., Bradley, D.G., 2008. Detecting the effects of selection at the population level in six bovine immune genes. BMC Genet 9, 62.
Fried, B., 1994. Metacercarial excystment of trematodes. Advances in parasitology 33, 91-144.
Fuertes, M., Manga-Gonzalez, Y., Benavides, J., Gonzalez-Lanza, M.C., Giraldez, F.J., Mezo, M., Gonzalez-Warleta, M., Fernandez, M., Regidor-Cerrillo, J., Castano, P., Royo, M., Ortega-Mora, L.M., Perez, V., Ferreras, M.C., 2015. Immunohistochemical study and mRNA cytokine profile of the local immune response in cattle naturally infected with Calicophoron daubneyi. Veterinary parasitology.
Furst, T., Keiser, J., Utzinger, J., 2012. Global burden of human food-borne trematodiasis: a systematic review and meta-analysis. The Lancet. Infectious diseases 12, 210-221.
Garza-Cuartero, L., O'Sullivan, J., Blanco, A., McNair, J., Welsh, M., Flynn, R.J., Williams, D., Diggle, P., Cassidy, J., Mulcahy, G., 2016. Fasciola hepatica Infection Reduces Mycobacterium bovis burden and Mycobacterial Uptake and Suppresses the Pro-inflammatory Response. Parasite immunology.
Ginetsinkaya, T.A., 1988. Trematodes, their Life Cycles, Biology and Evolution. Amerind Publishing Co., Pvt. Ltd, New Delhi.

Glauert, A.M., Lammas, D.A., Duffus, W.P., 1985. Ultrastructural observations on the interaction in vitro between bovine eosinophils and juvenile Fasciola hepatica. Parasitology 91 (Pt 3), 459-470.
Godfray, H.C., Beddington, J.R., Crute, I.R., Haddad, L., Lawrence, D., Muir, J.F., Pretty, J., Robinson, S., Thomas, S.M., Toulmin, C., 2010. Food security: the challenge of feeding 9 billion people. Science 327, 812-818.
Golbar, H.M., Izawa, T., Juniantito, V., Ichikawa, C., Tanaka, M., Kuwamura, M., Yamate, J., 2013. Immunohistochemical characterization of macrophages and myofibroblasts in fibrotic liver lesions due to Fasciola infection in cattle. The Journal of veterinary medical science / the Japanese Society of Veterinary Science 75, 857-865.
Golden, O., Flynn, R.J., Read, C., Sekiya, M., Donnelly, S.M., Stack, C., Dalton, J.P., Mulcahy, G., 2010. Protection of cattle against a natural infection of Fasciola hepatica by vaccination with recombinant cathepsin L1 (rFhCL1). Vaccine 28, 5551-5557.
Gonzalez-Lanza, C., Manga-Gonzalez, Y., Del-Pozo-Carnero, P., Hidalgo-Arguello, R., 1989. Dynamics of elimination of the eggs of Fasciola hepatica (Trematoda, Digenea) in the faeces of cattle in the Porma Basin, Spain. Veterinary parasitology 34, 35-43.
Gordon, D., Zadoks, R., Skuce, P., Sargison, N., 2012. Confirmation of triclabendazole resistance in liver fluke in the UK. Vet Rec 171, 159-160.
Gordon, D.K., Roberts, L.C., Lean, N., Zadoks, R.N., Sargison, N.D., Skuce, P.J., 2013. Identification of the rumen fluke, Calicophoron daubneyi, in GB livestock: possible implications for liver fluke diagnosis. Veterinary parasitology 195, 65-71.
Gordon, S., 2003. Alternative activation of macrophages. Nature reviews. Immunology 3, 23-35.
Graczyk, T.K., Fried, B., 1999. Develoopment of *Fasciola hepatica* in the Intermediate Host, In: Dalton, J.P. (Ed.) Fasciolosis. CABI publishing.
Guasconi, L., Chiapello, L.S., Masih, D.T., 2015. Fasciola hepatica excretory-secretory products induce CD4+T cell anergy via selective up-regulation of PD-L2 expression on macrophages in a Dectin-1 dependent way. Immunobiology 220, 934-939.
Guasconi, L., Serradell, M.C., Garro, A.P., Iacobelli, L., Masih, D.T., 2011. C-type lectins on macrophages participate in the immunomodulatory response to Fasciola hepatica products. Immunology 133, 386-396.
Guasconi, L., Serradell, M.C., Masih, D.T., 2012. Fasciola hepatica products induce apoptosis of peritoneal macrophages. Veterinary immunology and immunopathology 148, 359-363.
Hacariz, O., Sayers, G., Flynn, R.J., Lejeune, A., Mulcahy, G., 2009. IL-10 and TGF-beta1 are associated with variations in fluke burdens following experimental fasciolosis in sheep. Parasite immunology 31, 613-622.
Hacariz, O., Sayers, G., Mulcahy, G., 2011. A preliminary study to understand the effect of Fasciola hepatica tegument on naive macrophages and humoral responses in an ovine model. Veterinary immunology and immunopathology 139, 245-249.
Hamilton, C.M., Dowling, D.J., Loscher, C.E., Morphew, R.M., Brophy, P.M., O'Neill, S.M., 2009. The Fasciola hepatica tegumental antigen suppresses dendritic cell maturation and function. Infection and immunity 77, 2488-2498.
Hansen, R.D., Trees, A.J., Bah, G.S., Hetzel, U., Martin, C., Bain, O., Tanya, V.N., Makepeace, B.L., 2011. A worm's best friend: recruitment of neutrophils by Wolbachia confounds eosinophil degranulation against the filarial nematode Onchocerca ochengi. Proc Biol Sci 278, 2293-2302.
Happich, F.A., Boray, J.C., 1969. Quantitative diagnosis of chronic fasciolosis. 1. Comparative studies on quantitative faecal examinations for chronic Fasciola hepatica infection in sheep. Australian veterinary journal 45, 326-328.
Hein, W.R., Mackay, C.R., 1991. Prominence of gamma delta T cells in the ruminant immune system. Immunology today 12, 30-34.
Heinrichs, A.J.S., L.A., 1914. Management of Dairy Heifers. The Pennsylvania State University, 35 p.
Heinrichs, J.C., Lammers, B., 1998. Monitorung Dairy Heifer Growth. The Pennsylvania State University.
Hillyer, G.V., 1980. Isolation of Fasciola hepatica tegument antigens. Journal of clinical microbiology 12, 695-699.
Hillyer, G.V., Apt, W., 1997. Food-borne trematode infections in the Americas. Parasitology Today 13, 87-88.
Hodgkinson, J., Cwiklinski, K., Beesley, N.J., Paterson, S., Williams, D.J., 2013. Identification of putative markers of triclabendazole resistance by a genome-wide analysis of genetically recombinant Fasciola hepatica. Parasitology 140, 1523-1533.

Hoek, A., Rutten, V.P., Kool, J., Arkesteijn, G.J., Bouwstra, R.J., Van Rhijn, I., Koets, A.P., 2009. Subpopulations of bovine WC1(+) gammadelta T cells rather than CD4(+)CD25(high) Foxp3(+) T cells act as immune regulatory cells ex vivo. Veterinary research 40, 6.

Honer, M.R., 1965a. The interpretation of faecal egg-counts. 3. The influence of the age of the host on Fasciola hepatica egg-counts in cattle. Zeitschrift fur Parasitenkunde (Berlin, Germany) 26, 221-229.

Honer, M.R., 1965b. The interpretation of faecal egg-counts. I. Daily variations in Fasciola hepatica egg-counts in cattle. Zeitschrift fur Parasitenkunde (Berlin, Germany) 26, 143-155.

Hope, J.C., Kwong, L.S., Thom, M., Sopp, P., Mwangi, W., Brown, W.C., Palmer, G.H., Wattegedera, S., Entrican, G., Howard, C.J., 2005. Development of detection methods for ruminant interleukin (IL)-4. J Immunol Methods 301, 114-123.

Hori, S., Sakaguchi, S., 2004. Foxp3: a critical regulator of the development and function of regulatory T cells. Microbes and infection / Institut Pasteur 6, 745-751.

Howell, A., Baylis, M., Smith, R., Pinchbeck, G., Williams, D., 2015. Epidemiology and impact of Fasciola hepatica exposure in high-yielding dairy herds. Preventive veterinary medicine 121, 41-48.

Howell, A., Mugisha, L., Davies, J., LaCourse, E.J., Claridge, J., Williams, D.J., Kelly-Hope, L., Betson, M., Kabatereine, N.B., Stothard, J.R., 2012. Bovine fasciolosis at increasing altitudes: parasitological and malacological sampling on the slopes of Mount Elgon, Uganda. Parasites & vectors 5, 196.

Hoyle, D.V., Taylor, D.W., 2003. The immune response of regional lymph nodes during the early stages of Fasciola hepatica infection in cattle. Parasite immunology 25, 221-229.

Issia, L., Pietrokovsky, S., Sousa-Figueiredo, J., Stothard, J.R., Wisnivesky-Colli, C., 2009. Fasciola hepatica infections in livestock flock, guanacos and coypus in two wildlife reserves in Argentina. Veterinary parasitology 165, 341-344.

Japa, O., Hodgkinson, J.E., Emes, R.D., Flynn, R.J., 2015. TGF-beta superfamily members from the helminth Fasciola hepatica show intrinsic effects on viability and development. Veterinary research 46, 29.

Jarrett, W.F., Jennings, F.W., Mc, I.W., Mulligan, W., Urquhart, G.M., 1960. Immunological studies on Dictyocaulus viviparus infection; immunity produced by the administration of irradiated larvae. Immunology 3, 145-151.

Jones, T.C., King, N.W., Hunt, R.D., 1997. Veterinary pathology. Baltimore, Md : Williams & Wilkins, 1997. 6th ed.

Kajugu, P.E., Hanna, R.E., Edgar, H.W., McMahon, C., Cooper, M., Gordon, A., Barley, J.P., Malone, F.E., Brennan, G.P., Fairweather, I., 2015. Fasciola hepatica: Specificity of a coproantigen ELISA test for diagnosis of fasciolosis in faecal samples from cattle and sheep concurrently infected with gastrointestinal nematodes, coccidians and/or rumen flukes (paramphistomes), under field conditions. Veterinary parasitology 212, 181-187.

Kelch, W.J., Coles, B.M., Reynolds, G.E., Bailey, D.E., 1977. Suspected infectious necrotic hepatitis (black disease) in Oregon cattle. Journal of the American Veterinary Medical Association 171, 431-432.

Kendall, S., 1953. The life-history of Limnaea truncatula under laboratory conditions. Journal of helminthology 27, 17-28.

Kenyon, F., Sargison, N.D., Skuce, P.J., Jackson, F., 2009. Sheep helminth parasitic disease in south eastern Scotland arising as a possible consequence of climate change. Veterinary parasitology 163, 293-297.

Kerney, M.P., 1999. Atlas of the land and freshwater molluscs of Britain and Ireland. Harley Books, Colchester.

Khan, M.K., Sajid, M.S., Khan, M.N., Iqbal, Z., Arshad, M., Hussain, A., 2011. Point prevalence of bovine fascioliasis and the influence of chemotherapy on the milk yield in a lactating bovine population from the district of Toba Tek Singh, Pakistan. Journal of helminthology 85, 334-338.

Kuerpick, B., Fiedor, C., von Samson-Himmelstjerna, G., Schnieder, T., Strube, C., 2012. Bulk milk-estimated seroprevalence of Fasciola hepatica in dairy herds and collecting of risk factor data in East Frisia, northern Germany. Berliner und Munchener tierarztliche Wochenschrift 125, 345-350.

Kuerpick, B., Schnieder, T., Strube, C., 2013. Evaluation of a recombinant cathepsin L1 ELISA and comparison with the Pourquier and ES ELISA for the detection of antibodies against Fasciola hepatica. Veterinary parasitology 193, 206-213.

Kwong, L.S., Hope, J.C., Thom, M.L., Sopp, P., Duggan, S., Bembridge, G.P., Howard, C.J., 2002. Development of an ELISA for bovine IL-10. Vet Immunol Immunopathol 85, 213-223.

Lee, C.G., Cho, S.H., Lee, C.Y., 1995. Metacercarial production of Lymnaea viridis experimentally infected with Fasciola hepatica. Veterinary parasitology 58, 313-318.

Lesschen, J.P., van den Berg, M., Westhoek, H.J., Witzke, H.P., Oenema, O., 2011. Greenhouse gas emission profiles of European livestock sectors. Animal Feed Science and Technology 166–167, 16-28.
Levine, N.D., 1970. Integrated control of snails. American zoologist 10, 579-582.
Lopez-Diaz, M.C., Carro, M.C., Cadorniga, C., Diez-Banos, P., Mezo, M., 1998. Puberty and serum concentrations of ovarian steroids during prepuberal period in Friesian heifers artificially infected with Fasciola hepatica. Theriogenology 50, 587-593.
Losada, H., Hirsch, M., Guzman, P., Fonseca, F., Hofmann, E., Alanis, M., 2015. Fascioliasis simulating an intrahepatic cholangiocarcinoma-Case report with imaging and pathology correlation. Hepatobiliary surgery and nutrition 4, E1-7.
LVPD 2015. Liverpool Veterinary Diagnostics Price List 2015/16, Liverpool, U.o., ed.
Maggioli, G., Acosta, D., Silveira, F., Rossi, S., Giacaman, S., Basika, T., Gayo, V., Rosadilla, D., Roche, L., Tort, J., Carmona, C., 2011. The recombinant gut-associated M17 leucine aminopeptidase in combination with different adjuvants confers a high level of protection against Fasciola hepatica infection in sheep. Vaccine 29, 9057-9063.
Mallard, B.A., Dekkers, J.C., Ireland, M.J., Leslie, K.E., Sharif, S., Vankampen, C.L., Wagter, L., Wilkie, B.N., 1998. Alteration in immune responsiveness during the peripartum period and its ramification on dairy cow and calf health. J Dairy Sci 81, 585-595.
Martin, I., Caban-Hernandez, K., Figueroa-Santiago, O., Espino, A.M., 2015. Fasciola hepatica fatty acid binding protein inhibits TLR4 activation and suppresses the inflammatory cytokines induced by lipopolysaccharide in vitro and in vivo. Journal of immunology (Baltimore, Md. : 1950) 194, 3924-3936.
Martinez-Perez, J.M., Robles-Perez, D., Rojo-Vazquez, F.A., Martinez-Valladares, M., 2012. Comparison of three different techniques to diagnose Fasciola hepatica infection in experimentally and naturally infected sheep. Veterinary parasitology 190, 80-86.
Martinez-Sernandez, V., Muino, L., Perteguer, M.J., Garate, T., Mezo, M., Gonzalez-Warleta, M., Muro, A., Correia da Costa, J.M., Romaris, F., Ubeira, F.M., 2011. Development and evaluation of a new lateral flow immunoassay for serodiagnosis of human fasciolosis. PLoS neglected tropical diseases 5, e1376.
Mas-Coma, S., Funatsu, I.R., Bargues, M.D., 2001. Fasciola hepatica and lymnaeid snails occurring at very high altitude in South America. Parasitology 123 Suppl, S115-127.
Mas-Coma, S., Valero, M.A., Bargues, M.D., 2009. Chapter 2. Fasciola, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. Advances in parasitology 69, 41-146.
Masih, D.T., Cervi, L., Casado, J.M., 1996. Modification of accessory activity of peritoneal cells from Fasciola hepatica infected rats. Veterinary immunology and immunopathology 53, 257-268.
McCann, C.M., Baylis, M., Williams, D.J., 2010. Seroprevalence and spatial distribution of Fasciola hepatica-infected dairy herds in England and Wales. Vet Rec 166, 612-617.
McCole, D.F., Doherty, M.L., Baird, A.W., Davies, W.C., McGill, K., Torgerson, P.R., 1999. T cell subset involvement in immune responses to Fasciola hepatica infection in cattle. Parasite immunology 21, 1-8.
McCole, D.F., Doherty, M.L., Baird, A.W., Davis, W.C., McGill, K., Torgerson, P.R., 1998a. Concanavalin A-stimulated proliferation of T cell subset-depleted lymphocyte populations isolated from Fasciola hepatica-infected cattle. Veterinary immunology and immunopathology 66, 289-300.
McCole, D.F., Doherty, M.L., Torgerson, P.R., Baird, A.W., 1998b. Local immune responses in colon from cattle infected with Fasciola hepatica. International journal for parasitology 28, 1733-1737.
McNeilly, T.N., Rocchi, M., Bartley, Y., Brown, J.K., Frew, D., Longhi, C., McLean, L., McIntyre, J., Nisbet, A.J., Wattegedera, S., Huntley, J.F., Matthews, J.B., 2013. Suppression of ovine lymphocyte activation by Teladorsagia circumcincta larval excretory-secretory products. Veterinary research 44, 70.
Meeusen, E., Lee, C.S., Rickard, M.D., Brandon, M.R., 1995. Cellular responses during liver fluke infection in sheep and its evasion by the parasite. Parasite immunology 17, 37-45.
Menard, A., Agoulon, A., L'Hostis, M.L., Rondelaud, D., Collard, S., Chauvin, A., 2001. Myocastor coypus as a reservoir host of Fasciola hepatica in France. Veterinary research 32, 499-508.
Mendes, E.A., Mendes, T.A., dos Santos, S.L., Menezes-Souza, D., Bartholomeu, D.C., Martins, I.V., Silva, L.M., Lima Wdos, S., 2013. Expression of IL-4, IL-10 and IFN-gamma in the liver tissue of cattle that are naturally infected with Fasciola hepatica. Veterinary parasitology 195, 177-182.
Met-Office, U. 2013. Summer 2013 (Met Office).

Mezo, M., Gonzalez-Warleta, M., Carro, C., Ubeira, F.M., 2004. An ultrasensitive capture ELISA for detection of Fasciola hepatica coproantigens in sheep and cattle using a new monoclonal antibody (MM3). The Journal of parasitology 90, 845-852.
Mezo, M., Gonzalez-Warleta, M., Castro-Hermida, J.A., Manga-Gonzalez, M.Y., Peixoto, R., Mas-Coma, S., Valero, M.A., 2013. The wild boar (Sus scrofa Linnaeus, 1758) as secondary reservoir of Fasciola hepatica in Galicia (NW Spain). Veterinary parasitology 198, 274-283.
Mezo, M., Gonzalez-Warleta, M., Castro-Hermida, J.A., Muino, L., Ubeira, F.M., 2011. Association between anti-F. hepatica antibody levels in milk and production losses in dairy cows. Veterinary parasitology 180, 237-242.
Millar, M., Colloff, A., Scholes, S., 2012. Disease associated with immature paramphistome infection. The Veterinary record 171, 509.
Mitchell, G.B., Armour, J., Ross, J.G., Halliday, W.G., 1981. Successful passive transfer of resistance to Fasciola hepatica infection in rats by immune serum and transfer factor. Research in veterinary science 30, 246-247.
Mitchell, G.B., Maris, L., Bonniwell, M.A., 1998. Triclabendazole-resistant liver fluke in Scottish sheep. Vet Rec 143, 399.
Molina-Hernandez, V., Mulcahy, G., Perez, J., Martinez-Moreno, A., Donnelly, S., O'Neill, S.M., Dalton, J.P., Cwiklinski, K., 2015. Fasciola hepatica vaccine: we may not be there yet but we're on the right road. Veterinary parasitology 208, 101-111.
Moll, L., Gaasenbeek, C.P., Vellema, P., Borgsteede, F.H., 2000. Resistance of Fasciola hepatica against triclabendazole in cattle and sheep in The netherlands. Veterinary parasitology 91, 153-158.
Monaghan, M.L., Doherty, M.L., Collins, J.D., Kazda, J.F., Quinn, P.J., 1994. The tuberculin test. Veterinary microbiology 40, 111-124.
Mottier, L., Alvarez, L., Fairweather, I., Lanusse, C., 2006. Resistance-induced changes in triclabendazole transport in Fasciola hepatica: ivermectin reversal effect. The Journal of parasitology 92, 1355-1360.
Mulcahy, G., Joyce, P., Dalton, J.P., 1999. Immunology of *Fasciola hepatica* infection, In: Dalton, J.P. (Ed.) Fasciolosis. CABI publishing.
Mulcahy, G., O'Connor, F., McGonigle, S., Dowd, A., Clery, D.G., Andrews, S.J., Dalton, J.P., 1998. Correlation of specific antibody titre and avidity with protection in cattle immunized against Fasciola hepatica. Vaccine 16, 932-939.
Murphy, K.P., Travers, P., Walport, M., Janeway, C.A., Mowat, A., Weaver, C., 2012. Janeway's immunobiology. New York ; Garland Science, 2012. 8th ed.
NADIS 2016. the NADIS parasite forecast (National Animal Disease Information Service).
Nansen, P., 1975. Resistance in cattle to Fasciola hepatica induced by gamma-ray attenuated larvae: results from a controlled field trial. Research in veterinary science 19, 278-283.
NOAH, 2016. Compendium of Data Sheets for Animal Medicines.
Novobilsky, A., Hoglund, J., 2015. First report of closantel treatment failure against Fasciola hepatica in cattle. International journal for parasitology. Drugs and drug resistance 5, 172-177.
O'Neill, S.M., Brady, M.T., Callanan, J.J., Mulcahy, G., Joyce, P., Mills, K.H.G., Dalton, J.P., 2000. Fasciola hepatica infection downregulates Th1 responses in mice. Parasite immunology 22, 147-155.
O'Neill, S.M., Mills, K.H., Dalton, J.P., 2001. Fasciola hepatica cathepsin L cysteine proteinase suppresses Bordetella pertussis-specific interferon-gamma production in vivo. Parasite immunology 23, 541-547.
Oblitas, P.L.O., 1997. Humoral immune response to Fasciola hepatica in experimentally infected calves and in cattle naturally exposed to fasciolosis in Cajamarca, Peru. University of Liverpool,
Olaechea, F., Lovera, V., Larroza, M., Raffo, F., Cabrera, R., 2011. Resistance of Fasciola hepatica against triclabendazole in cattle in Patagonia (Argentina). Veterinary parasitology 178, 364-366.
Oldham, G., Williams, L., 1985. Cell mediated immunity to liver fluke antigens during experimental Fasciola hepatica infection of cattle. Parasite immunology 7, 503-516.
Oliveira, L.J., Hansen, P.J., 2008. Deviations in populations of peripheral blood mononuclear cells and endometrial macrophages in the cow during pregnancy. Reproduction (Cambridge, England) 136, 481-490.
Ollerenshaw, C., 1966. The approach to forecasting the incidence of fascioliasis over England and Wales 1958–1962. Agricultural Meteorology 3, 35-53.
Olsen, A., Frankena, K., Bodker, R., Toft, N., Thamsborg, S.M., Enemark, H.L., Halasa, T., 2015. Prevalence, risk factors and spatial analysis of liver fluke infections in Danish cattle herds. Parasites & vectors 8, 160.

Ortiz, P., Scarcella, S., Cerna, C., Rosales, C., Cabrera, M., Guzman, M., Lamenza, P., Solana, H., 2013. Resistance of Fasciola hepatica against Triclabendazole in cattle in Cajamarca (Peru): a clinical trial and an in vivo efficacy test in sheep. Veterinary parasitology 195, 118-121.

Ortiz, P.L., Claxton, J.R., Clarkson, M.J., McGarry, J., Williams, D.J., 2000. The specificity of antibody responses in cattle naturally exposed to Fasciola hepatica. Veterinary parasitology 93, 121-134.

Over, H.J., 1982. Ecological basis of parasite control: trematodes with special reference to fascioliasis. Veterinary parasitology 11, 85-97.

Overend, D.J., Bowen, F.L., 1995. Resistance of Fasciola hepatica to triclabendazole. Australian veterinary journal 72, 275-276.

Piedrafita, D., Parsons, J.C., Sandeman, R.M., Wood, P.R., Estuningsih, S.E., Partoutomo, S., Spithill, T.W., 2001. Antibody-dependent cell-mediated cytotoxicity to newly excysted juvenile Fasciola hepatica in vitro is mediated by reactive nitrogen intermediates. Parasite immunology 23, 473-482.

Pritchard, G.C., Forbes, A.B., Williams, D.J., Salimi-Bejestani, M.R., Daniel, R.G., 2005. Emergence of fasciolosis in cattle in East Anglia. The Veterinary record 157, 578-582.

Prowse, R.K., Chaplin, P., Robinson, H.C., Spithill, T.W., 2002. Fasciola hepatica cathepsin L suppresses sheep lymphocyte proliferation in vitro and modulates surface CD4 expression on human and ovine T cells. Parasite immunology 24, 57-66.

Puente, G.L., 1997. Acute and subacute Fasciolosis of alpacas (Lama pacos) and treatment with triclabendazole. Tropical animal health and production 29, 31-32.

Radostits, O.M., Littlejohns, I.R., 1988. New concepts in the pathogenesis, diagnosis and control of diseases caused by the bovine viral diarrhea virus. Can Vet J 29, 513-528.

Raghupathy, R., 1997. Th1-type immunity is incompatible with successful pregnancy. Immunology today 18, 478-482.

Rahko, T., 1969. The pathology of natural Fasciola hepatica infection in cattle. Pathologia veterinaria 6, 244-256.

Randell, W.F., Bradley, R.E., 1980. Effects of hexachlorethane on the milk yields of dairy cows in north Florida infected with Fasciola hepatica. American journal of veterinary research 41, 262-263.

Rapsch, C., Schweizer, G., Grimm, F., Kohler, L., Bauer, C., Deplazes, P., Braun, U., Torgerson, P.R., 2006. Estimating the true prevalence of Fasciola hepatica in cattle slaughtered in Switzerland in the absence of an absolute diagnostic test. International journal for parasitology 36, 1153-1158.

Rauw, W.M., 2012. Immune response from a resource allocation perspective. Frontiers in Genetics 3, 267.

Reichel, M.P., 2002. Performance characteristics of an enzyme-linked immunosorbent assay for the detection of liver fluke (Fasciola hepatica) infection in sheep and cattle. Veterinary parasitology 107, 65-72.

Relf, V., Good, B., McCarthy, E., de Waal, T., 2009. Evidence of Fasciola hepatica infection in Radix peregra and a mollusc of the family Succineidae in Ireland. Veterinary parasitology 163, 152-155.

Roberts, H., 1968. Observations on experimental acute fascioliasis in sheep. British Veterinary Journal 124, 433-450.

Robinson, M.W., Donnelly, S., Hutchinson, A.T., To, J., Taylor, N.L., Norton, R.S., Perugini, M.A., Dalton, J.P., 2011. A family of helminth molecules that modulate innate cell responses via molecular mimicry of host antimicrobial peptides. PLoS pathogens 7, e1002042.

Robinson, M.W., Menon, R., Donnelly, S.M., Dalton, J.P., Ranganathan, S., 2009. An integrated transcriptomics and proteomics analysis of the secretome of the helminth pathogen Fasciola hepatica: proteins associated with invasion and infection of the mammalian host. Molecular & cellular proteomics : MCP 8, 1891-1907.

Rosbottom, A., Gibney, E.H., Guy, C.S., Kipar, A., Smith, R.F., Kaiser, P., Trees, A.J., Williams, D.J., 2008. Upregulation of cytokines is detected in the placentas of cattle infected with Neospora caninum and is more marked early in gestation when fetal death is observed. Infect Immun 76, 2352-2361.

Rosbottom, A., Guy, C.S., Gibney, E.H., Smith, R.F., Valarcher, J.F., Taylor, G., Williams, D.J., 2007. Peripheral immune responses in pregnant cattle following Neospora caninum infection. Parasite Immunol 29, 219-228.

Ross, J., 1968a. A comparison of the resistance status of hosts to infection with Fasciola hepatica. Vet. Med. Rev., Leverkusen, 96-105.

Ross, J.G., 1968b. The life span of *Fasciola hepatica* in cattle. Veterinary Record 82, 587-589.

Ross, J.G., 1970. The economics of Fasciola hepatica infections in cattle. The British veterinary journal 126, xiii-xv.
Ross, J.G., Todd, J.R., Dow, C., 1966. Single experimental infections of calves with the liver fluke, Fasciola Hepatica (Linnaeus 1758). Journal of comparative pathology 76, 67-81.
Rowcliffe, S.A., Ollerenshaw, C.B., 1960. Observations on the bionomics of the egg of Fasciola hepatica. Annals of tropical medicine and parasitology 54, 172-181.
Ryan, J. 2008. Endotoxins and Cell Culture, Tchnical Bulletin. In TC.305 (Corning Life Sciences).
Sadeghi-Hashjin, G., Naem, S., 2001. Parasiticidal effects of peroxynitrite on ovine liver flukes in vitro. Journal of helminthology 75, 73-76.
Salimi-Bejestani, M.R., Cripps, P., Williams, D.J., 2008. Evaluation of an ELISA to assess the intensity of Fasciola hepatica infection in cattle. The Veterinary record 162, 109-111.
Salimi-Bejestani, M.R., Daniel, R.G., Felstead, S.M., Cripps, P.J., Mahmoody, H., Williams, D.J., 2005a. Prevalence of Fasciola hepatica in dairy herds in England and Wales measured with an ELISA applied to bulk-tank milk. Vet Rec 156, 729-731.
Salimi-Bejestani, M.R., McGarry, J.W., Felstead, S., Ortiz, P., Akca, A., Williams, D.J., 2005b. Development of an antibody-detection ELISA for Fasciola hepatica and its evaluation against a commercially available test. Research in veterinary science 78, 177-181.
Sanin, D.E., Mountford, A.P., 2015. Sm16, a major component of Schistosoma mansoni cercarial excretory/secretory products, prevents macrophage classical activation and delays antigen processing. Parasites & vectors 8, 1.
Sargison, N., 2006. New worming guidelines: Quarantine anthelmintic treatments. Livestock 11, 57-61.
Sargison, N.D., Scott, P.R., 2011. Diagnosis and economic consequences of triclabendazole resistance in Fasciola hepatica in a sheep flock in south-east Scotland. Vet Rec 168, 159.
Sargison, N.D., Wilson, D.J., Penny, C.D., Bartley, D.J., 2010. Unexpected production loss caused by helminth parasites in weaned beef calves. The Veterinary record 167, 752-754.
Savage, J., Meaney, M., Brennan, G.P., Hoey, E., Trudgett, A., Fairweather, I., 2013. Effect of the P-glycoprotein inhibitor, R(+)-verapamil on the drug susceptibility of a triclabendazole-resistant isolate of Fasciola hepatica. Veterinary parasitology 195, 72-86.
Schweizer, G., Braun, U., Deplazes, P., Torgerson, P.R., 2005. Estimating the financial losses due to bovine fasciolosis in Switzerland. Vet Rec 157, 188-193.
SCOPS 2012. Sustainable control of Parasites in Sheep. In Control of Worms Sustainably (SCOPS).
Seddon, M.B., Kebapçı, U., Van Damme, D. 2015. *Galba truncatula* In The IUCN Red List of Threatened Species 2015: e.T155730A85693575.
Sekiya, M., Zintl, A., Doherty, M.L., 2013. Bulk milk ELISA and the diagnosis of parasite infections in dairy herds: a review. Irish veterinary journal 66, 14.
Seo, K.S., Davis, W.C., Hamilton, M.J., Park, Y.H., Bohach, G.A., 2009. Development of monoclonal antibodies to detect bovine FOXP3 in PBMCs exposed to a staphylococcal superantigen. Veterinary immunology and immunopathology 128, 30-36.
Serradell, M.C., Guasconi, L., Cervi, L., Chiapello, L.S., Masih, D.T., 2007. Excretory-secretory products from Fasciola hepatica induce eosinophil apoptosis by a caspase-dependent mechanism. Veterinary immunology and immunopathology 117, 197-208.
Sibille, P., Tliba, O., Boulard, C., 2004. Early and transient cytotoxic response of peritoneal cells from Fasciola hepatica-infected rats. Veterinary research 35, 573-584.
Simm, G., 2000. Genetic improvement of cattle and sheep. Tombridge : Farming Press, 2000.
Smeal, M.G., Hall, C.A., 1983. The activity of triclabendazole against immature and adult Fasciola hepatica infections in sheep. Australian veterinary journal 60, 329-331.
Smith, A.M., Dowd, A.J., Heffernan, M., Robertson, C.D., Dalton, J.P., 1993. Fasciola hepatica: a secreted cathepsin L-like proteinase cleaves host immunoglobulin. International journal for parasitology 23, 977-983.
Smith, D., Newlands, G., Dobson, R., Kahn, L., Fitzpatrick, J., Beiser, B., 2015. First season performance of Barbervax, a novel vaccine for Haemonchus contortus, now commercially available in Australia. In: 25th International Conference of the World Association for the Advancement of Veterinary Parasitology, Liverpool, Thursday 20th August, p. 227.
Smyth, J.D., Halton, D.W., 1983. The physiology of trematodes. CUP Archive.
Soares, M.P., da Silva, S.S., Nizoli, L.Q., Felix, S.R., Schild, A.L., 2007. Chronic fascioliasis in farmed and wild greater rheas (Rhea americana). Veterinary parasitology 145, 168-171.
Spithill, T.W., Smooker, P.M., Sexton, J.L., Bozas, E., Morrison, C.A., Creaney, J., Parsons, J.C., 1999. Development of vaccines against *Fasciola hepatica*, In: Dalton, J.P. (Ed.) Fasciolosis.CABI publishing, pp. 377-410.

Suhardono, Roberts, J.A., Copeman, D.B., 2006. Optimization of the procedure for counting the eggs of Fasciola gigantica in bovine faeces. Tropical animal health and production 38, 383-387.
Suweis, S., Carr, J.A., Maritan, A., Rinaldo, A., D'Odorico, P., 2015. Resilience and reactivity of global food security. Proc Natl Acad Sci U S A 112, 6902-6907.
Sykes, A.R., Coop, R.L., Rushton, B., 1980. Chronic subclinical fascioliasis in sheep: effects on food intake, food utilisation and blood constituents. Research in veterinary science 28, 63-70.
Symons, L., Boray, J., 1967. Anaemia of ovine fascioliasis. Vet. Rec 81, 130.
Taira, N., Yoshifuji, H., Boray, J.C., 1997. Zoonotic potential of infection with Fasciola spp. by consumption of freshly prepared raw liver containing immature flukes. International journal for parasitology 27, 775-779.
Taylor, M.A., 2012. Emerging parasitic diseases of sheep. Veterinary parasitology 189, 2-7.
Taylor, M.A., Coop, R.L., Wall, R.L., 2016. Veterinary parasitology. [electronic book]. Chichester, West Sussex ; John Wiley and Sons, Inc., 2016. 4th ed.
Thienpont, D., Rochette, R., Vanparjis, O.F.J., 1986. Diagnosing Helminthiasis by Coprological Examination, 2nd Edition. Janssen Research Foundation, Beerse, Belgium.
Thomas, I., Coles, G.C., Duffus, K., 2000. Triclabendazole-resistant Fasciola hepatica in southwest Wales. Vet Rec 146, 200.
Tizard, I.R., 2013. Veterinary immunology. St. Louis, Mo. : Elsevier/Saunders, c2013. 9th ed.
Tkalcevic, J., Ashman, K., Meeusen, E., 1995. Fasciola hepatica: rapid identification of newly excysted juvenile proteins. Biochemical and biophysical research communications 213, 169-174.
Tkalcevic, J., Brandon, M.R., Meeusen, E.N., 1996. Fasciola hepatica: rapid switching of stage-specific antigen expression after infection. Parasite immunology 18, 139-147.
Toet, H., Piedrafita, D.M., Spithill, T.W., 2014. Liver fluke vaccines in ruminants: strategies, progress and future opportunities. International journal for parasitology 44, 915-927.
Toner, E., Brennan, G.P., Hanna, R.E., Edgar, H.W., Fairweather, I., 2011. Disruption of egg formation by Fasciola hepatica following treatment in vivo with triclabendazole in the sheep host. Veterinary parasitology 177, 79-89.
Torgerson, P., Claxton, J., 1999. Epidemiology and Control, In: Dalton, J.P. (Ed.) Fasciolosis. CABI publishing.
Tort, J., Brindley, P.J., Knox, D., Wolfe, K.H., Dalton, J.P., 1999. Proteinases and associated genes of parasitic helminths. Advances in parasitology 43, 161-266.
Turner, J., Howell, A., McCann, C., Caminade, C., Bowers, R.G., Williams, D., Baylis, M., 2016. A model to assess the efficacy of vaccines for control of liver fluke infection. Scientific reports 6, 23345.
Turner, K., Armour, J., Richards, R.J., 1984. Anthelmintic efficacy of triclabendazole against Fasciola hepatica in sheep. The Veterinary record 114, 41-42.
United Nations 2013. World Population Prospects: The 2012 Revision, Key Findings and Advance Tables, Department of Economic and Social Affairs, P.D., ed.
Vaessen, M.A., Veling, J., Frankena, K., Graat, E.A., Klunder, T., 1998. Risk factors for Salmonella dublin infection on dairy farms. The Veterinary quarterly 20, 97-99.
van Dijk, J., Sargison, N.D., Kenyon, F., Skuce, P.J., 2010. Climate change and infectious disease: helminthological challenges to farmed ruminants in temperate regions. Animal : an international journal of animal bioscience 4, 377-392.
Van Milligen, F.J., Cornelissen, J.B., Bokhout, B.A., 1999. Protection against Fasciola hepatica in the intestine is highly correlated with eosinophil and immunoglobulin G1 responses against newly excysted juveniles. Parasite immunology 21, 243-251.
Van Milligen, F.J., Cornelissen, J.B., Hendriks, I.M., Gaasenbeek, C.P., Bokhout, B.A., 1998. Protection of Fasciola hepatica in the gut mucosa of immune rats is associated with infiltrates of eosinophils, IgG1 and IgG2a antibodies around the parasites. Parasite immunology 20, 285-292.
Vazquez, A.A., Lounnas, M., Sanchez, J., Alba, A., Milesi, A., Hurtrez-Bousses, S., 2016. Genetic and infective diversity of the liver fluke Fasciola hepatica (Trematoda: Digenea) from Cuba. Journal of helminthology, 1-7.
Vercruysse, J., Claerebout, E., 2001. Treatment vs non-treatment of helminth infections in cattle: defining the threshold. Veterinary parasitology 98, 195-214.
Villa-Mancera, A., Mendez-Mendoza, M., 2012. Protection and antibody isotype responses against Fasciola hepatica with specific antibody to pIII-displayed peptide mimotopes of cathepsin L1 in sheep. Vet J 194, 108-112.
Villa-Mancera, A., Reynoso-Palomar, A., Utrera-Quintana, F., Carreon-Luna, L., 2014. Cathepsin L1 mimotopes with adjuvant Quil A induces a Th1/Th2 immune response and confers significant protection against Fasciola hepatica infection in goats. Parasitology research 113, 243-250.

Vukman, K.V., Adams, P.N., Metz, M., Maurer, M., O'Neill, S.M., 2013a. Fasciola hepatica tegumental coat impairs mast cells' ability to drive Th1 immune responses. Journal of immunology (Baltimore, Md. : 1950) 190, 2873-2879.

Vukman, K.V., Adams, P.N., O'Neill, S.M., 2013b. Fasciola hepatica tegumental coat antigen suppresses MAPK signalling in dendritic cells and up-regulates the expression of SOCS3. Parasite immunology 35, 234-238.

Walker, S.M., Johnston, C., Hoey, E.M., Fairweather, I., Borgsteede, F.H., Gaasenbeek, C.P., Prodohl, P.A., Trudgett, A., 2011. Potential role of hares in the spread of liver fluke in the Netherlands. Veterinary parasitology 177, 179-181.

Walsh, K.P., Brady, M.T., Finlay, C.M., Boon, L., Mills, K.H., 2009. Infection with a helminth parasite attenuates autoimmunity through TGF-beta-mediated suppression of Th17 and Th1 responses. Journal of immunology (Baltimore, Md. : 1950) 183, 1577-1586.

Walton, C., Wright, W.R., 1926. Hydrogen-ion concentration and the distribution of Limnaea truncatula and L. peregra, with a note bearing on mosquitoes. Parasitology 18, 363-367.

Wang, Y., Zhou, H., Shen, Y., Wang, Y., Wu, W., Liu, H., Yuan, Z., Xu, Y., Hu, Y., Cao, J., 2015. Impairment of dendritic cell function and induction of CD4(+)CD25(+)Foxp3(+) T cells by excretory-secretory products: a potential mechanism of immune evasion adopted by Echinococcus granulosus. BMC immunology 16, 44.

Wedin, W.F., Jacobson, N.L., Hodgson, H.J. 1975. Utilizing plant and animal resources in producing human food, pp. 667-686.

Wegmann, T.G., Lin, H., Guilbert, L., Mosmann, T.R., 1993. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? Immunology today 14, 353-356.

Wessa, P. 2014. Maximum-likelihood Negative Binomial Distribution Fitting (v1.0.3). In Free Statistics Software (v1.1.23-r7) (Office for Research Development and Education).

WHO 2010. First WHO report on neglected tropical diseases: working to overcome the global impact of neglected tropical diseases, Diseases, W.D.o.C.o.N.T., ed. (WHO press).

Williams, D.J., Guy, C.S., McGarry, J.W., Guy, F., Tasker, L., Smith, R.F., MacEachern, K., Cripps, P.J., Kelly, D.F., Trees, A.J., 2000. Neospora caninum-associated abortion in cattle: the time of experimentally-induced parasitaemia during gestation determines foetal survival. Parasitology 121 (Pt 4), 347-358.

Williams, D.J.L., Howell, A., Graham-Brown, J., Kamaludeen, J., Smith, D., 2014. Liver fluke–an overview for practitioners. CATTLE PRACTICE 22, 238-244.

Wrigley, J., McArthur, M., McKenna, P.B., Mariadass, B., 2006. Resistance to a triple combination of broad-spectrum anthelmintics in naturally-acquired Ostertagia circumcincta infections in sheep. New Zealand veterinary journal 54, 47-49.

Yalav, O., Yagmur, O., Ulku, A., Akcam, A.T., Sonmez, H., 2012. A rare cause of obstructive jaundice: Fasciola hepatica mimicking cholangiocarcinoma. The Turkish journal of gastroenterology : the official journal of Turkish Society of Gastroenterology 23, 604-607.

Zafra, R., Perez-Ecija, R.A., Buffoni, L., Moreno, P., Bautista, M.J., Martinez-Moreno, A., Mulcahy, G., Dalton, J.P., Perez, J., 2013. Early and late peritoneal and hepatic changes in goats immunized with recombinant cathepsin L1 and infected with Fasciola hepatica. Journal of comparative pathology 148, 373-384.

Zimmerman, G.L., Kerkvliet, N.I., Brauner, J.A., Cerro, J.E., 1983. Modulation of host immune responses by Fasciola hepatica: responses by peripheral lymphocytes to mitogens during liver fluke infections of sheep. The Journal of parasitology 69, 473-477.