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The prevalence and production effects of liver fluke (*Fasciola hepatica*) in New Zealand cattle including evaluation of diagnostic tests.

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

In

Veterinary Science

At Massey University, Palmerston North, New Zealand

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2023

Abstract

The liver fluke *Fasciola hepatica*, infects cattle worldwide and is considered a parasite of regional importance in New Zealand although the impact on milk production in that country have not been studied.

The test characteristics of two antibody detection ELISAs; IDEXX ELISA (IDEXX Fasciolosis Verification) and an In-House assay using unrefined excretory secretory antigens, plus a coproantigen ELISA (Bio K 201–Monoscreen AgELISA) and faecal egg counts (FEC) were assessed against the gold standard of total fluke counts in naturally infected cattle (cows=29, steers=10). Vat milk of dairy herds on the West Coast of the South Island was assessed for liver fluke infection using the IDEXX ELISA in the autumn, near the end of one lactation (n=430), and spring, near the beginning of the subsequent lactation(n=403). A total of 156 questionnaires determining awareness of liver fluke infection and drenching practices were completed. A cross sectional study of 11 herds (n=1314 cows) in autumn and a longitudinal study of 4 herds (n=485 cows) in spring and autumn used the IDEXX ELISA (measured as SP%) on serum to analyse associations between liver fluke infection and milk production parameters in individual cows. A subset of cows was also faecal sampled for coproantigen and FEC analysis.

Notably, a negative linear effect of the $\log_e$(total fluke count+1) on liveweight (p=0.02) was found and the coproantigen values showed a significant (p=0.01) quadratic effect for $\log_e$(total fluke count+1). The survey showed that infection of herds at a level likely to cause production losses on the West Coast is common, with regional clustering. Milk Fat % decreased 0.0004% points for every 1SP% increase (p=0.004), being 0.05 %points lower for cows with SP%150 than cows with SP%30, and 0.22 %points (p=0.014) lower in cows where SP% increased from ≤30 to ≥150 during lactation compared that those remained ≤30 with an economic cost of $55.19 per infected cow. Of the tests compared, the IDEXX ELISA was superior to the In-House ELISA for sensitivity (Se) and specificity (Sp) but the coproantigen ELISA had the highest Se (96%) and Sp (96%).

Overall, liver fluke infection was common in dairy cows but the infection intensity was low, nevertheless a small effect on MF% was determined.

Acknowledgements

I will be forever grateful to my chief supervisor, Professor Bill Pomroy for encouraging me to investigate the liver fluke *Fasciola hepatica*, joining me on trips to the West Coast, mentoring and encouraging me to stay on track to complete this thesis (your last one?). You have always been a great support to me through this long journey of completing a PhD on a part time basis. I owe my knowledge of molecular biology, the development and optimisation of ELISA as a diagnostic tool and good laboratory rigour to Associate Professor Laryssa Howe. You have also helped to keep my chin up when my assays did not always perform as expected. Associate Professor Kevin Lawrence was instrumental in guiding me using R as a statistical tool, model development and particularly the use of Bayesian latent class modelling to investigate the performance of diagnostic tests in New Zealand cattle. He led me carefully along the path from a novice understanding of statistics to understanding aspects of Bayesian statistics. Thanks also to Dr Ian Scott who completed my supervisory team, you managed to give me support through a period of significant change in the parasitology department at Massey University.

Thank you to the Massey University Ethics Committee for considering the proposals to sample animals and survey farmers.

This thesis involved the processing of many faecal, milk and serum samples. To all the laboratory staff who helped particularly, Barbara Adlington, Anne Tunnicliffe, Liz Burrows and Brittnee Southland, thank you for welcoming me into the lab and helping me when I was snowed under with samples.

Without the cooperation of Westland Milk Co-operative sending milk samples and posting out survey forms and ANZCO Stratford allowing me access to the livers of cattle, the completion of this thesis would not have been possible. Special thanks must be made to the farmers who welcomed me onto their farms to sample cattle and analyse their milk production records, as without your support these on farm studies would not have been possible. Thanks also to Simon Hewitt and the team at West Coast vets in Hokitika for allowing the use of your facilities to process and store samples as well as sampling some of the heifers.

I would like to acknowledge PGG Wrightson Ltd for supporting me, initially financially, and also allowing me to find time to complete this thesis while working full-time.

Finally, I acknowledge to incredible support from my wife Amanda as without this I would not have been able to make those trips to the West Coast, up to Massey University and then sit down and complete this thesis. Now we can have some more time together.

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List of Abbreviations

Abbreviation	Meaning	Abbreviation	Meaning
%	percentage	HAU	haemagglutination units
+/-	plus and, or minus	kg/d	kilograms per day
<	less than	kDa	kilodaltons
>	greater than	kg	kilograms
≤	less than or equals	LAMP	loop-mediated isothermal amplification
≥	greater than or equals	LCM	latent class model
®	registered trademark	LIC	Livestock Improvement Corporation
°C	degrees Celsius	LSTM	Liverpool School of Tropical Medicine
2X	two times	M	month
Ab	antibody	MA	Massachusetts
Ab- or -Ab	antibody negative	MAC	mixed age cow
ABZ	albendazole	MAP	mean annual precipitation
Ag	antigen	MAT	mean annual temperature
Ag- or -Ab	antigen negative	MF	milk fat
Ag+ or +Ag	antigen positive	ML	milk lactose
Ag+ or +Ag	antigen positive	mL	millilitre
BME	bulk milk ELISA		
BTM	bulk tank milk	mm	millimetre
BZ	benzimidazole	ML	milk lactose (kg or %)
CET	controlled efficacy test	MF	milk fat (kg or %)
CL	cathepsin L	MP	milk protein (kg or %)
CLOR	clorsulon	MS	milk solids (kg)
CLOS	closantel	NAIT	National Animal Identification & Tracing
cm	centimetre	NC	negative control
Cneg	negative control	NE	net extinction
Cpos	positive control	NIT	nitroxynil
df	degrees of freedom	NIWA	National Institute of Water and Atmospheric Research
ECM	energy corrected milk	NPV	negative predictive value
ELISA	enzyme linked immunosorbent assay	NZ	New Zealand
epg	eggs per gram	OD	optical density
ES	excretory-secretory	ODR	optical density ratio
ESA	excretory-secretory antigen	P	probability value
et al.	and others	PC	positive control
FE	facial eczema	PCR	polymerase chain reaction
FECRT	faecal egg count reduction test	P_{dry}	precipitation in the driest month
g	gram	pH	potential of hydrogen
GDD	growing degree-day	PPV	positive predictive value
GGT	gamma-glutamyl transferase	P_{sdry}	precipitation in the driest summer month
GIS	geographical information systems	P_{swet}	precipitation in the wettest summer month

Abbreviation	Meaning	Abbreviation	Meaning
GST	glutathione S-transferase	pt	post-treatment
h	hours	Pthreshold	precipitation threshold
HA	haemagglutination	Pwdry	precipitation in the driest winter month
Pwwet	precipitation in the wettest winter month	Tmon10	number of months with air temperature >10 oC
SCC	somatic cell counts	µg	micrograms
Se	selenium	µm	micrometre
Se	sensitivity	USA	United States of America
SOP	standard operating procedure	VCS	virtual climate stations
Sp	specificity	WAAVP	World Association for the Advancement of Veterinary Parasitology
SP%	sample to positive percent	wpi	weeks post-infection
steer	castrated male	y	Year
TCBZ	triclabendazole	ZnSo4	zinc sulphate
Tcold	air temperature of the coldest month		

Chapter 1. Introduction and Literature review

1.1 Introduction

Fasciola hepatica (liver fluke) is a trematode parasite of farmed livestock with a worldwide distribution and which is also capable of infecting humans. The presence of suitable intermediate host snails and definitive hosts with patent infections are required for liver fluke to maintain itself, with the grazing of pastures suited to these intermediate host snails allowing the possibility of new infections over prolonged periods and possibly year-round. The adaptability of the liver fluke is reflected in the ability to exploit different intermediate host snails around the world, making it a difficult parasite to manage.

There is no debate about the capacity of the liver fluke to cause harm in the definitive host resulting in welfare issues and interfering with normal functioning of the liver. In New Zealand there have been only a limited number of studies on this parasite. At present liver fluke is known to be endemic in some regions but very little is known of the distribution of the intermediate host snails, a critical aspect of the parasite establishing in new regions as climate warms. While liver fluke is considered an animal health risk by farmers and their veterinarians, there is little knowledge of its impact on animal health and production under New Zealand pastoral farming conditions.

The overarching aim of this thesis was to investigate the impacts of *Fasciola hepatica* on milk production in dairy herds on the West Coast of New Zealand. However, it was first necessary to evaluate diagnostic tools for *Fasciola hepatica*. The results from these studies would then provide a basis to identify further research questions on the impact of this parasite in the wider New Zealand context.

In Chapter 2 the focus was on defining and characterising the usefulness of diagnostic tests that would then be used as tools for future studies in West Coast cattle. Several of the available diagnostic tests were investigated to determine their test characteristics to detect and quantify liver fluke in cattle with chronic, natural infections. The tests included animal liveweights, liver pathology scoring, faecal egg counts, serum antibody and coproantigen ELISAs and be assessed against the gold standard of total fluke count. It was also the intention to adapt an antibody ELISA assay using unrefined excretory-secretory antigens to reduce costs of later studies, so this was included in the analysis. Studies on this Taranaki farm would also provide an interesting case report of the production impacts on beef cattle on a New Zealand farm with endemic liver fluke infection.

In Chapter 3 the focus was on investigating the prevalence of infection of the dairy herds in the study region, the West Coast of the South Island. The West Coast was chosen in part due to most herds supplying the same milk processing plant to facilitate access to samples. This resulted in a more complete analysis of liver fluke infection in that region. This region is also considered to be a low risk for both facial eczema (pithomycotoxicosis) and theileriosis, two important confounding factors in some dairy regions. Bulk milk samples collected in the autumn, near the end of lactation and spring, and near the beginning of the subsequent lactation in the same calendar year were analysed using a commercial antibody ELISA. A short postal survey was also sent to farmers to determine their awareness of liver fluke infection on their dairy farms on the West Coast and the anthelmintic practices used.

In Chapter 4 the focus was tightened to investigate any impacts of liver fluke infection on individual cow milk production parameters. To achieve this, eleven herds were enrolled in a cross-sectional study. Cows from these 11 herds were serum sampled near the end of that season's lactation,

analysed using a commercial antibody ELISA and milk production measurements from the herd testing events used to investigate any associations. In three herds, faecal samples were also analysed using faecal egg counts and a coproantigen ELISA. Where available, younger cattle on the farms were also serum sampled to investigate the antibody concentration in different age cohorts.

In Chapter 5 the focus was extended in a longitudinal study where cows from four herds were serum sampled in the spring and autumn, with the effect of any change in infection status on milk production investigated. This study also provided sufficient samples to complete a study on the test characteristics of the In-House ELISA using the IDEXX ELISA as the gold standard. The variation of the results based on season of sampling are reported.

In Chapter 6 the focus returned to further evaluating three commonly available diagnostic tests using Bayesian latent class modelling. These models were used to test three diagnostic tests in two herds, and two diagnostic tests in one herd, evaluating the predicted liver fluke prevalence in each herd and test characteristics. The implication of the outcomes of these models for the veterinarian when choosing which diagnostic tests to use will be discussed.

1.2 Literature Review

1.2.1 The New Zealand Dairy Industry

New Zealand is a large export earner of dairy products, grossing $19 billion in 2021, being the leading export industry and contributing 9% of the gross domestic product (New Zealand: export revenue of dairy industry 2023 | Statista). At present dairy cattle are farmed in all provinces of New Zealand (Alvarez-Mercado *et al.* 2015).

In the 2018/19 milk season 5 million cows produced 21.2 billion litres of milk containing 1.88 billion kilograms (kg) of milk solids comprising 1.06 billion kg milkfat and 0.83 billion kg of milk protein. There were 11,372 herds averaging 435 cows per herd (LIC and NZ 2019). The West Coast (West Coast of the South Island) comprised 3.3% of the herds (372) and 3.1% of the cows (153,077) with an average herd size of 411 cows producing 2.7% of the national milk solids (51.7 million kg) (LIC and NZ 2019). The milk fat and milk protein production of West Coast cows are less than the national herd (Table 1.1).

Table 1.1 New Zealand and West Coast average cow milk solids production 2018/19 (LIC and NZ)

	District	Number of dairy herds	Cows per herd	Average kg milk solids per cow	Average kg milk fat per cow	Average kg milk protein per cow
New Zealand		11,372	435	381	214	167
West Coast		372	411	338	191	147
	Buller	128	400	330	186	144
	Grey	89	470	348	196	151
	Westland	155	388	338	192	146

New Zealand dairy farms are typified by seasonal milk production utilising grass grazed *in situ*, with varying volumes of supplements fed over the year, dependant on the on-farm facilities, and access to supplementary feeds. Farm calendars are based on the grass growth pattern of the farm. Calving most commonly occurs in July and August when temperatures and soil moisture support pasture growth (predominantly ryegrass and white clover mixes). Lactation ceases for spring calving herds in the late autumn or early winter due to low pasture growth, the need to allow involution of mammary issue and gain and body condition in cows prior to calving. A small proportion of farms will calve all or part of the herd in the autumn to provide milk over the winter period, generally for local consumption. Surplus grass growth is harvested and stored as silage to feed in times when grass growth does not meet cow demand. Forage crops may be planted for grazing at specific times such as a summer when rain may be deficient or for grazing during the non-lactating period.

All cows are registered on a national animal identification system (NAIT) using an electronic ear tag. In addition, a separate herd number associated with a separate numbered tag is invariably used. The latter are used for day-to-day management and are updated every year. Thus, the same numbered tag may be used to identify different cows in different seasons.

Farmers can record data from each cow including production data (milk volume, fat and protein content), animal health events (mastitis treatment, reproduction interventions, anthelmintic administration), and animal parameters (body condition score, weight) using electronic recording

systems. Milk production characteristics of each cow can be monitored using a herd testing service provided commercially by companies and this was undertaken for 3.7 million cows which were tested at least once in the 2018/19 season (LIC and NZ 2019). This involves a technician attaching equipment to each cluster of milk cups in the milking parlour to monitor the volume of milk produced by each cow. A milk sample from each cow is also analysed in the laboratory for fat and protein content as well as calculating the somatic leucocyte cell count from the leucocyte cell content. Farmers may use this data for making farm management decisions such as culling, semen selection for mating and long-acting antibiotic therapy over the non-lactating (dry) period. On an annual basis an average of 72.8% of herds performed at least one herd test nationally and for West Coast herds this was slightly lower at 65.9% (LIC and NZ 2019). Up to five herd tests may be undertaken over a season. The mean cow milk production values for dairy cows farmed in the West Coast are somewhat similar to New Zealand as a whole (Table 1.2).

Table 1.2 Estimated annual mean values for components of milk per cow for New Zealand and the West Coast based on herd tests over 2018/19

Region	Milk (litres)	Milkfat (kg)	Milkfat (%)	Milk protein (kg)	Milk protein (%)	Milk solids (kg)	Milk solids (%)
New Zealand	4,359	206	4.72	167	3.84	373	8.56
West Coast	4,060	202	4.98	161	3.97	364	8.95

In New Zealand, the farmer will have a contract to supply milk to one processor. Milk from the cows is stored in a refrigerated stainless-steel silo referred to as the vat. A tanker truck will usually arrive on the farm each day and collect the milk from the vat. At this time a sample of the vat milk is collected and analysed by the processor. This sample can be used for herd level testing for specific disease (eg. Bovine viral diarrhoea virus) or antibodies to indicate infection with parasites (eg. liver fluke or ostertagiosis).

Payments to seasonal supply farmers are based on the "A+B±C" system, which incorporates payments for milkfat (A) and protein (B) with adjustments for milk volume (C). New Zealand is a major exporter of dairy products, and these are predominantly sold in a dry form. Thus, the focus for production is the milk solids (A+B) and not the volume. The New Zealand dairy herd is a mixture of Holstein-Friesian / Jersey crossbred cows (48.5% of the national herd), Holstein-Friesian (33.1%), Jersey (8.6%), Ayrshire (0.5%) and other breeds (9.3%) (LIC and NZ 2019). The mature bodyweight of each breed is different, and farmers may choose to have cows that are of a bodyweight that does not cause unnecessary soil and pasture damage in their environment. Friesian cows consume more dry matter, produce more litres of milk with a lower milk fat percentage per cow than Jersey cows. The cross bred offspring have milk production parameters in between the two pure breeds although there is considerable variation within each breed (Palladino *et al.* 2010). West Coast and Tasman herds have a greater percentage of Jersey cows than the national herd at the expense of Holstein-Friesian cows (Table 1.3).

Table 1.3 Genetic characteristics of dairy herds and variance between West Coast and Tasman herds from the national average

	Holstein-Friesian	Holstein-Friesian / Jersey Cross	Jersey	Ayrshire	Other
New Zealand	33.1%	48.5%	8.6%	0.5%	9.3%
West Coast and Tasman	16.5% (-16.6%)	52.5% (+4%)	17.9% (+9.3%)	0.5%	12.9% (+3.6%)

1.3 Some factors impacting milk production on the West Coast

The West Coast of the South Island is characterised by frequent, heavy rainfall which maintains soil moisture and supports pasture growth. One of the major constraints of milk production on farms is the availability of sufficient, high-quality feed (S Hewitt pers comms).

There are a number of disease conditions that impact milk production in New Zealand dairy cows. These include Jöhnes disease, facial eczema and oriental theileriosis. Jöhnes disease is a contagious disease of cattle and sheep and is caused by the bacteria *Mycobacterium avium subspecies paratuberculosis*. Infection leads to thickening of the terminal ileum and disrupts the absorption of nutrients leading to decreased milk production; it is present in many regions of New Zealand. Clinical disease is uncommon and tends to affect individual cows, characterised by watery diarrhoea and weight loss (Beef and Lamb 2016). Testing of the milk from cows is an aid to determining those that are likely to suffer clinical disease or be spreading the bacteria, identifying these cows for culling.

Pithomycotoxicosis , colloquially referred to as facial eczema (FE), is a common cause of pathological change to the livers of farmed ruminants in the North Island and northern South Island of New Zealand (Laven *et al.* 2020). It is the result of the toxin sporisdesmin in the spores of the saprophytic fungus *Pithomyces chartarum* being absorbed from the digesta in the intestines causing considerable oxidative damage to the biliary system of the liver. The fungus grows prolifically in dead matter on pasture when night temperatures are greater than 12°C and there is sufficient moisture present (Cuttance *et al.* 2016). The West Coast of the South Island is not considered to be a high-risk region for FE although the disease has been detected in herds close to Hokitika.

The tick-borne intracellular parasite of red blood cells, *Theileria orientalis* is present in New Zealand as Type 1 (Chitose), Type 2 (Ikeda), Type 3 (Buffeli) and Type 5 genotypes. Type 2, in particular, causes infectious bovine anaemia. The parasite is spread by the cattle tick *Haemaphysalis longicornis* with the subclinical effects having significant impact, resulting in anaemia in up to 26% of cows in recently infected herds (Lawrence *et al.* 2021b). The West Coast of the South Island is not considered to be suitable for the maintenance of the cattle tick, although they have been identified in both Karamea and Murchison (Lawrence *et al.* 2021a) and hence theileriosis is not considered endemic on the West Coast. The movement of infected cattle from regions in New Zealand where theileriosis is endemic to the West Coast could introduce the parasite to a herd but without the cattle tick being present the parasite cannot be transmitted.

1.4 Fasciola hepatica

1.4.1 History

Our knowledge of liver fluke is the accumulation of over 100 years of active research with major break throughs including the identification of the intermediate host and the development of climate models to predict risk periods of fascioliasis.

The following history is as noted by Andrews (Andrews 1999). Liver rot in sheep was first referenced in historical texts as far back as 1200AD in the "Black Book of Chirk". It was thought to be associated with toxins within something that the sheep were eating. In 1523 a reference is made of liver fluke by Sir Anthony Fitzherbert citing an Italian physician Fanansi Gabucinus who described worms resembling pumpkin seeds in the blood vessels of the livers of sheep and goats. In 1688 Francesco Redi showed that this parasite laid eggs, challenging the thought that the disease was caused by eating particular plants and also debunked the idea of spontaneous generation of parasites. He was also the first to publish a sketch of a liver fluke. Dutchman Johann Swammerdam in 1758 observed *living things* while dissecting a snail, which he thought were not of snail origin and identified them as cercariae of some trematode. Otto Muller in 1773 identified microscopic, tadpole like creatures swimming in the water of ponds which he called cercariae and Ludwig Bojanus noted the resemblance between redia, cercariae and flukes and in 1858 observed the birth of cercariae from redia. In 1880 Professor James Simmonds reported failing to find a single fluke after infecting sheep 6 months prior with thousands of fluke eggs, thus identifying that an intermediate stage of the parasite life cycle must exist. Miracidia were observed to penetrate into snails by Guido Wagener in 1857. Thus collectively, all of these pieces of the lifecycle jigsaw were progressively being observed but the lack of finding the intermediate host was halting further developments. This finally changed when David Weinland, in 1857, found "cercariae sacs" in *Galba truncatula* snails and also noted that cercariae had a strong drive to leave water and climb onto objects. He conjectured that these cercariae encysted on grass and were eaten by sheep. However, it is Algernon Thomas in 1881 who is credited as the first person to discover that *G. truncatula* was an intermediate host for *F. hepatica*. To complete the life cycle, it was Dimitry Sinistin in 1914 who observed young flukes emerging from their cysts in the intestine, penetrating the gut wall, and migrating to the liver via the peritoneal cavity.

1.4.2 Liver fluke in New Zealand

The only species of liver fluke found in New Zealand is *Fasciola hepatica*. This species has a worldwide distribution in temperate climatic zones, being first reported in New Zealand in Te Hauke, Hawkes Bay in 1896 (Harris *et al.* 1986) and was most likely introduced by sheep imported from Australia. The presence of the native intermediate host snail *Australopeplea tomentosa* enabled the liver fluke to establish and spread to other regions of both the North and South Islands by 1945. In 1969 the presence of the exotic snail *Pseudosuccinea columella* was first identified in New Zealand and is thought to be responsible for significant geographical spread of liver fluke both within existing endemic regions and creating new endemic regions. This snail has a much wider ecological niche and appears to have replaced *A. tomentosa* in some regions (Charleston 1997). Few recent studies have been conducted to determine the regional prevalence of liver fluke in New Zealand (Charleston and

McKenna 2002), and with slaughter of animals outside of the region they were born and raised being commonplace, the data collected may not be of much value.

1.4.3 Lifecycle

Fasciola hepatica has an indirect life cycle typical of most (digenetic) trematodes. It is one of two species of the genus *Fasciola* with *Fasciola gigantica* being the other. These two species are generally allopatric with *F. gigantica* being found in tropical regions and only some minor overlap at the climatic margins of the two species. They also utilise different intermediate hosts.

Fasciola hepatica can parasitise a range of different definitive hosts including all ruminants, and to a lesser extent humans. In New Zealand liver fluke has been recorded in cattle, sheep, goats, red deer, fallow deer, llamas, pigs, possums and humans. The development of liver fluke in the intermediate and definitive hosts is variable both within and between definitive host species, and between different isolates of flukes (Fairweather 2011).

The lifecycle of the liver fluke is complex involving a definitive host, environmental stages and a mud snail as an intermediate host. It can be broken into 5 main stages:

- Passage of the eggs from the host to the outside environment and development of miracidia within them
- Miracidia hatching and finding the intermediate host
- Development and multiplication of the parasite within the intermediate host
- Cercariae emerging from the snail intermediate host
- Ingestion of the metacercariae by the host and successful establishment in the bile ducts.

1.4.4 Development and hatching of the miracidia.

Developing eggs are very prone to desiccation (Ollerenshaw 1971), thus any eggs passed into a dry environment are unlikely to develop. They also need a good supply of oxygen and thus complete development and hatching of the egg will not occur until the egg is freed from the faecal pile (Rowcliffe and Ollerenshaw 1960b). For embryonation to occur a minimum temperature of 10°C is required (Table 1.4), but takes 6 months to develop at this temperature, whereas at 30°C the process takes only 8 days. Eggs can withstand a very wide range of pH but the optimal is neutral at 7 (Al-Habbib and Grainger 1983).

Several studies of egg survival and development have been conducted in the field in various places around the world, and all show similar seasonal patterns, although, eggs will continue development over the warmer winter in Australia (Boray 1963) but not in the cooler winter of Great Britain (Rowcliffe and Ollerenshaw 1960a). The mortality rate of eggs greatly increases when mean temperature is below 10°C, with only embryonated eggs able to overwinter (Luzon-Pena *et al.* 1994). At the other extreme, egg mortality was 100% after 24 days at 37°C (Rowcliffe and Ollerenshaw 1960b) so very high temperatures are also poor for survival. Eggs in faeces survived at least 10 weeks in summer and 6 months in winter in the UK whereas if the faeces were allowed to desiccate, eggs did not survive beyond 2.5 weeks in the summer and 7.5 weeks in winter. Egg survival may be higher in

winter but development to hatching did not occur at temperature <10°C. In contrast, in the summer adequate moisture will be the limiting factor (Rowcliffe and Ollerenshaw 1960b). At an average temperature of 16°C egg development takes 2-3 months while at 23-26°C it was only 2-3 weeks (Andrews *et al.* 2022). In the northern regions of the West Coast the daily mean maximum temperature can rise above 16°C for seven months of the year and above 23°C for two months while the more southern regions are cooler, not rising above 20°C. Hence conditions are potentially suitable for development of eggs for a large portion of the year.

Vibration, increased light and a sudden change in temperature stimulate egg hatching and miracidium release (Wilson 1968; Smith and Grenfell 1984) and mass hatching of eggs can be induced in the laboratory using a strong light and sudden immersion is a large volume of cold tap water. This mimics a "fresh" flow of water coming down a stream and flooding the surrounding muddy areas, a feature common on the West Coast.

Table 1.4 Effect of Temperature on the development of the liver fluke at constant temperatures (Ollerenshaw 1971)

Temperature (c)	Development of fluke eggs (days)	Development in snails (days)	Development from egg to infection on herbage (weeks)
10	No development	No development	-
15	40	82	17
17.5	27	53	11
20	20	40	8.5
22.5	15	34	7
25	11	25	5
27.5	10	22	4.5

Miracidium swim at 1mm/second toward light (Wilson and Denison 1970) surviving in constant water temperature for 40 hours at 6°C, 20 hours at 10°C and 10 hours at 20°C (Smith and Grenfell 1984). Being phototrophic they swim randomly near the surface of the water until coming close to the host snail and then penetrating the foot.

1.4.5 Development in the intermediate host snail.

Around the world a variety of different snails in the family Lymnaeidae may act as suitable intermediate hosts. These snails inhabit freshwater environments and are commonly described as mud snails. In New Zealand there is one suitable indigenous Lymnaeid snail, *Australopeplea tomentosa* (previously named *Lymnaea tomentosa*) and the introduced *Pseudosuccinea columella* (previously named *Lymnaea columella*), a North American species now endemic in New Zealand being first detected in 1940 (Pullan *et al.* 1972). *Pseudosuccinea columella* has a much greater distribution and appears to have replaced *A. tomentosa* in some regions (Charleston 1997). These mud snails prefer habitats where the feed source of benthic algae grow; shallow water, muddy substratum and limited shading (Pullan *et al.* 1972). Harris (1974a) concluded that *A. tomentosa* preferred margins of still water such as spring fed marshes and whilst *P. columella* also preferred still water, it was more likely to exist within bodies of water than *A. tomentosa*. The ubiquitous drainage ditches and small streams throughout the West Coast are likely to provide a suitable habitat for both species.

Pseudosuccinea columella produce three times more eggs than *A. tomentosa* and can produce eggs at temperatures as low as 2°C. The minimum time from oviposition to hatching was 6.5 days at 25-27°C. At 22°C it takes 30 days for *P. columella* to reach sexual maturity (Harris and Charleston 1980).

A comparison of the infectivity of these two species concluded that *A. tomentosa* produced more cercariae than *P. columella* (Boray 1978) although a later study showed a large number of cercariae were produced in *P. columella* if the snails were infected early in life compared to no cercariae being produced when infection was attempted in mature adult snails (Boray *et al.* 1985). This has implications for the seasonality of cercariae being developed as infections are more likely in warmer weather when snails are reproducing.

In New Zealand *P. columella* is arguably the more important host due to its greater distribution and being a prolific breeder. The most recent detailed study of snail distribution in New Zealand was in the early 1970s and now needs repeating as changes in farming practice, irrigation and potential spread of snails by birds are likely to have made an impact. At the time of this last survey *P. columella* was not found on the West Coast of the South Island (Pullan *et al.* 1972).

In both laboratory and field studies *A. tomentosa* was not observed to copulate below 16°C or above 30°C (Boray 1969) which is a common temperature range on the West Coast implying that reproduction in snails is possible for many months of the year in that region. Hatching of eggs was first observed at 5°C with an upper limit of 25°C after which hatching became erratic. However, the highest hatch rates were at the lower temperatures in this range (Boray 1969). The seasonality of *A. tomentosa* and *P. columella* is poorly defined except that the warmer summer temperatures encourage more activity and a higher breeding rate in *P. columella* compared to *A. tomentosa* (Harris and Charleston 1977). Dry conditions over summer can also be damaging to snails. They can survive these conditions by aestivation, a survival strategy in which snails reduce their metabolic rate for weeks to months (Rubaba *et al.* 2016) during periods of excessive heat, dryness or when food is restricted. Snails may burrow into mud before aestivating making them difficult to locate during these periods (Lynch 1966) Survival over winter may be by hibernation, with egg laying resuming when temperatures warm up in spring/summer. Harris and Charleston (1976) observed on one farm in the Manawatu that very few snails survived over the winter, as their numbers declined to undetectable levels by late winter in four successive years. There have been no studies directly investigating either aestivation or hibernation for these intermediate host snails in New Zealand.

It has been observed that snail population density and intensity of infection in the definitive host are not directly related, but the proportion of immature snails in the population was more important since they are more prone to infection with miracidium (Boray 1963; Kendall and Ollerenshaw 1963; Harris and Charleston 1976; Boray *et al.* 1985). Nevertheless, large snails produce ten times more redia than small snails (Rowcliffe and Ollerenshaw 1960b) implying early infection but good snail growth due to a sufficient nutrient source whilst immature *Fasciola* stages develop to promote the production of greater numbers of cercariae. The use of molluscicides to interrupt the liver fluke lifecycle was not shown to be as effective as expected (Ollerenshaw 1971).

Further development of the lifecycle in the snail hosts causes considerable tissue damage (Rondelaud *et al.* 2007). The sporocyst travels to the digestive gland and grows before finally rupturing to release redia, a process which cause considerable damage to the digestive gland. Germinal balls then develop inside the migrating redia and cercariae exit these via a birth pore. Cercariae accumulate in the tissues

of the snail and then exit the snail five to twelve weeks after infection to encyst on vegetation or objects at the water surface being almost immediately infective to the definitive host (Andrews 1999). Clonal expansion occurs at each stage and in one study a single miracidium may develop into as many as 629 cercariae in *P. columella* 68-89 days after infection (Krull 1941) although higher numbers have been reported. Exposing snails with 20 miracidia in a lab at 22-24°C, 86% of *A. tomentosa* of Australian origin, 77% of *P. columella* of New Zealand origin and 72% of *P. columella* of Australian origin became infected with young sporocysts but only 32, 11.3 and 0.9% respectively produced cercariae. The number of metacercariae produced per 100 snails infected with miracidia was 15290, 8927 and 394. For Australia this confirmed *A. tomentosa* as the most significant intermediate host but the New Zealand isolate of *P. columella* was also able to produce metacercariae in reasonable numbers (Boray 1978). For New Zealand, the wider distribution of *P. columella* is also a factor. Another factor to consider is that multiple stages of the lifecycle can exist in the intermediate host and the cercariae do not all exit the host simultaneously, even if infected concurrently (Walker *et al.* 2006).

1.4.6 Cercaria migration from the intermediate host snail and metacercariae development.

Cercariae exit the snail and swim near the surface of the water for up to two hours and then settle onto vegetation or an object to which it attaches with its ventral sucker. The movement of both the miracidium and cercariae are thought to be due to innate patterns of behaviour at each stage of the life cycle which react to the presence of only a few signals (Thomas *et al.* 2002; Sukhdeo and Sukhdeo 2004) The tail is shed and the cercariae forms a white cyst which is immediately infective to the host. After a few days the cyst becomes yellow and hardens, the external layer of the metacercarial cyst adheres to inanimate objects such as grass and consists of tanned protein with an underlying fibrous layer while the inner cyst consists of four layers. Together these all provide significant protection with the cyst remaining infective even if the outer layer is removed (Andrews 1999).

Metacercariae survival is dependent on sufficient moisture and moderate temperatures. In one study 50% that encysted on herbage in September survived a UK winter to the following March (Rowcliffe and Ollerenshaw 1960b) but this was not observed in New Zealand (Harris and Charleston 1976) where none survived the winter in the Manawatu, thought to be due to low temperatures. Studies have shown that metacercariae are resistant to freezing between -2 and -10°C, while at 12-14°C 50% survived 6 months and 25% survived for 8 months but none survived more than six weeks at 25°C. Some regions of the West Coast experience overnight frosts where the air temperature will dip below 0°C during the winter but overall, the conditions would appear to be suitable for survival for at least as long as in the Manawatu if not longer. To date no studies have been conducted in these regions. Summer heat and drought caused high rates of metacercariae death (Boray and Enigk 1964; Love 2017) with these conditions being uncommon on the West Coast.

Metacercariae did not survive beyond two weeks in silage stored in good anaerobic conditions but silage with aerobic spoilage when the silage stack is not properly compacted or air is allowed access to the stack can result in metacercariae surviving for up to 10 weeks (John *et al.* 2020). Although it was unlikely that metacercariae would survive storage in properly made hay, it has been shown that hay that is harvested during rainy periods and stored at relatively high humidity allowed metacercarial survival for eight months over the winter (Boray 2005). Ensiling grass is a common method of

harvesting excess grass on the West Coast as this method does not need the longer periods without rain that hay requires.

1.4.7 Newly excysted juvenile flukes (NEJ).

Excystment occurs in the small intestine distal to the bile duct within three hours of ingestion and is triggered by exposure to high concentration of carbon dioxide, a reducing agent such as sodium dithionite or cysteine, temperature greater than 39°C and bile (Dixon 1966). This stimulates the active emergence of the metacercariae through the cyst wall. These newly excysted juvenile (NEJ) fluke penetrate the small intestinal wall and migrate across the abdominal cavity to reach the liver feeding on tissues during the journey and may penetrate other organs in transit (Dawes 1961;1963b; Burden *et al.* 1983). In endemic herds up to two percent of cattle had lung lesions caused by migrating flukes (Thornton 1949) and although rare, foetal infection has even occurred (Sinclair 1967). The flukes reach the liver after a migration of four to six days (Andrews 1999) and typically penetrate the left or ventral lobe, due to its proximity to the small intestine.

The young flukes penetrate and migrate through the liver tissue by secreting proteases to digest liver parenchyma (Dawes 1963a; Wilson *et al.* 1998), which causes considerable arteritis and marked fibrosis over the following three to four weeks. The flukes grow significantly during this period before reaching the bile ducts about seven weeks after infection. Many flukes can become trapped in the parenchyma with as few as 5% of metacercariae resulting in adults establishing in the bile ducts (Moazeni and Ahmadi 2016). In addition, decreasing parasite establishment was shown with successive artificial doses, indicating that cattle develop a form of resistance to infection (Ross 1966). This period of parenchyma damage is the period of greatest risk to the fluke life cycle as it may both harm the health of the host and reduce the growth rates of the flukes (Dawes 1964).

1.4.8 Development to adult fluke.

The flukes arrive at the bile ducts around seven weeks after infection in cattle (Moazeni and Ahmadi 2016). The resulting desquamation and ulceration of major bile ducts is more severe in cattle than other host species, with greater proliferation of granulation tissue (Sinclair 1967). The bile ducts in cattle thicken and calcium deposits form in the bile duct wall from 16-20 weeks after infection resulting in “stove pipe” liver, with the bile duct epithelium being ulcerated and haemorrhagic (Ross 1966; Behm and Sangster 1999).

Adult flukes respond to gastrointestinal hormones, when hormones are released by the host during eating. The flukes attach using their ventral sucker to prevent expulsion and when hormones are released at the end of eating the fluke releases the sucker and moves about the bile duct once more (Sukhdeo and Sukhdeo 1989; Thomas *et al.* 2002). Flukes feed on bile and blood.

The life expectancy of *F. hepatica* in cattle is not clearly determined. The livers of untreated 30 month old cattle can show severe signs of liver fluke infection without fluke being found indicating that they are not long lived (Dawes 1964). In cattle that were naturally infected and then removed from further fluke challenge or artificially infected, 75% of flukes did not survive beyond 5-21 months post infection, with some surviving to 26 months with lower numbers of flukes (Ross 1968). The ability of some flukes to live for more than two years is also reported in a Danish study (Takeuchi-Storm *et al.* 2018) and seemed to be related to low levels of infection or removal of further metacercarial challenge. Fluke

do not appear to be long lived in deer (Lamb *et al.* 2021), however, in sheep adult fluke are long lived (Ross 1968; Boray 1969) making this species an important source of fluke eggs on farms where they are co-grazed with cattle. To date, although some hypotheses have been proposed there is no firm explanation for the difference in fluke longevity between hosts. The longevity of adult fluke in cattle is particularly important in dairy cows on the West Coast with regards to understanding the epidemiology of infection. It is worth noting that there are very few sheep farmed on the West Coast of the South Island.

1.4.9 Reproduction

Fasciola flukes are simultaneous hermaphrodites, having two multibranched testis and one branched ovary with a common genital pore (Harris 1974b), but cross fertilisation is more common than self fertilisation (Beesley *et al*. 2017). The reproductive capacity is impressive. For example, a single rat infected with 13 adult flukes produced 13 million eggs over 3 years (Montgomerie 1931). In cattle, the flukes mature in the bile ducts and eggs can be detected in faeces 7 weeks after infection (Moazeni and Ahmadi 2016) with the accepted pre-patent period in cattle ranging from eight to ten weeks (Bouvry and Rau 1986; Boulard *et al.* 1995).

In sheep a single fluke can lay between 10,000 and 25,000 eggs per day (Happich and Boray 1969a) but in cattle the fecundity of the parasite is much lower. The fecundity of liver flukes from different regions of the world can also vary as seen in a study investigating the dynamics of the different stages of the life cycle (Walker *et al.* 2006).

1.5 Liver fluke infection risk modelling

Modelling strategies using climate factors including rainfall, evapotranspiration and temperature have been used to predict the risk of fasciolosis in farmed livestock in large geographical areas (Ollerenshaw and Rowlands 1959; Ollerenshaw and Smith 1969; Ross 1970; Malone *et al.* 1987; Malone *et al.* 1998). These models utilise historical data and related fluke incidence, but a weakness is that these predictions are at large geographical scales and may not reflect the actual risk at individual farms, due to a lack of precise climate data. Recently the ability to record climate data for much smaller geographical areas greatly improves the usefulness of these models for individual farmers. The development of geographical information systems (GIS) has enabled the use of spatial risk models, even when actual data has not been collected from that area, to both predict disease risk or describe spatial patterns of disease (Clements and Pfeiffer 2009). In New Zealand the National Institute of Water and Atmospheric Research (NIWA) have developed a series of virtual climate stations (VCS) that each cover a 5 square kilometre grid that estimate the weather data for that grid based on data from actual weather stations. These have been retrospectively estimated for each VCS back to 1972 (Haydock 2016). This data is more relevant to an individual farmer or smaller geographical regions like a river valley or small inland plain as are features of the West Coast.

Utilising the known parameters for development of each stage of the liver fluke lifecycle and activity of the intermediate host snails (Beltrame *et al.* 2018), Growing Degree-Day (GDD) models predict the number of infective metacercariae that will be present and therefore the risk of fasciolosis (Haydock *et al* 2016). This study determined that the West Coast has the highest risk value for fasciolosis of the 14 regions in New Zealand with predicted climate changes increasing this risk even more by 2090.

1.6 Liver fluke Prevalence around the world based on bulk milk ELISA testing

Liver fluke (*Fasciola hepatica*) is a worldwide problem (Vercruysse and Claerebout 2001; Pritchard *et al.* 2005; Charlier *et al.* 2014; Kelley *et al.* 2020; Waal. and Mehmood 2021). The worldwide prevalence of infection in dairy herds, detected using a bulk milk ELISA, ranges from 11-84%, with a production limiting infection (see Section 3.1.2) identified in 7-86% of infected farms (Table 1.5).

Table 1.5 Larger studies using bulk milk ELISA testing to demonstrate liver fluke prevalence with the percentage of herds infected and where measured, the percentage of herds with infection at a level expected to impact cow production. Test description: IDEXX= commercial kit IDEXX Fasciola verification, ES = excretory-secretory ELISA based on the LSTM ELISA or Biox ELISA, Svanovir = commercial kit Svanovir, Ildana = commercial kit Ildana.*

Author	Country and season	Year Sampled	Test	Infected herds	Production limiting	Number of herds
Salimi-Bejestani	Wales Winter	2005	ES		86%	445
McCann	Wales Winter	2006	ES	84%	84%	1,022
McCann	England Winter	2006	ES	72%	72%	2,108
Byrne	Ireland Spring	2016	IDEXX	74%	50%	1445
Byrne	Ireland Summer	2016	IDEXX	79%	46%	1447
Byrne	Ireland Autumn	2016	IDEXX	75%	51%	1437
Byrne	Ireland Winter	2016	IDEXX	75%	50%	1421
Kuerpick	Germany September	2008	ES		57%	868
Kuerpick	Germany November	2008	ES		54%	859
Kuerpick	Germany January	2008	ES		49%	861
Kuerpick	Germany September	2010	ES		50%	673
Kuerpick	Germany November	2010	ES		48%	669
Kuerpick	Germany January	2010	ES		45%	700
Salimi-Bejestani	England Winter	2003	ES		48%	623
McKay	Northland (NZ) Autumn	2008	IDEXX	71%	46%	250
Kuerpick	Germany Winter	2010	ES		45%	868
Kuerpick	Germany Summer	2006	IDEXX	54%	44%	757
Kuerpick	Germany Winter	2006	IDEXX	52%	44%	781
Bennema	Belgium Autumn	2006	ES		37%	1,762
Bennema	Belgium Autumn	2007	ES		40%	1,762
Bennema	Belgium Autumn	2008	ES		40%	1,762
Frey	Switzerland Winter	2014-16	IDEXX	41%	26%	1,036
Kuerpick	Germany	2008	ES		24%	20,749
Kostenberger	Austria Spring	2014	Svanovir	61%	22%	178
Kostenberger	Austria Spring	2015	Svanovir	46%	16%	178
Novobilsky	Sweden Autumn	2012	Svanovir	25%	7%	426
Bloemhoff	Ireland	2012	Ildana	52-76%		290
Villa-Mancera, A.	Mexico	2017	ES	64%		837
Fanke	Germany Autumn and Winter	2011	ES	33%		344
Arenal, A.	Cuba	2014	Ildana	82%		516
Conceicao	Portugal Spring to Summer	1997	ES	11%		151
Conceicao	Portugal Summer to Winter	1997	ES	23%		93
Conceicao	Portugal Spring to Summer	1998	ES	48%		468
Hoglund	Sweden Autumn	2008	IDEXX	8%		105

*Liverpool School of Tropical Medicine

Of note was that significant clustering of infected herds was observed in a Danish study and this supports the need for a population of intermediate host snails to enable endemic infection (Olsen *et al.* 2015).

There has only been one substantive slaughterhouse study on the prevalence of liver fluke in New Zealand (Charleston *et al.* 1990). In this study the prevalence of liver fluke infection in cattle detected at slaughter in Greymouth on the West Coast in the summer and autumn of two consecutive years was 32 and 35%, greater than that observed in Morewa, Northland where only 10% of livers were infected (Charleston *et al.* 1990). This higher prevalence indicates that the West Coast would be a suitable region for investigating the impacts of liver fluke infection.

1.7 Host response to the presence of *F. hepatica*

It should be noted that cattle appear to have some ability to resist infection as noted when serial dosing with metacercaria results in fewer flukes establishing and developing into adults. One potential explanation is that previous damage causes fibrosis of the liver capsule and tissue, which prevents entry and migration of juvenile flukes (Ross 1966; Boray *et al.* 1969). However, these findings were not supported in a separate study which found that heifers given two doses of metacercariae had similar fluke burdens to those naturally infected (Clery *et al.* 1996) although the number of animals in the study were small. The calcification of bile ducts in cattle is also suggested as a mechanism to reduce the lifespan of flukes in cattle (Ross 1966).

1.8 Production impacts of liver fluke infection

The liver pathology associated with liver fluke is a combination of parenchymal damage, during the migratory phase, and bile duct damage, during the adult stage. The impact of parenchymal and bile duct damage on the host depends on species infected. For example, it is rare in cattle but more common in sheep to see sudden death caused by the migratory phase. In high level infections many flukes become trapped in the parenchymal tissue with only 5% reaching the bile ducts (Ross 1966) and the local inflammatory response results in fibrosis of the parenchyma and bile ducts. The liver is a vital organ of the body conducting a range of normal physiological functions including metabolism of amino acids, carbohydrate, fat, urea, ketone, albumin and glutathione homeostasis and acting as the major detoxification centre of the body.

Determining the degree of impact of liver fluke infection on the individual or herd is problematic. The oral administration of single or multiple doses of high numbers of metacercariae to naïve animals, and monitoring the impact over a short period, does not reflect the dynamics of infection in animals on farms after natural exposure. Instead three categories of infection level, *therapeutic*, *production-based* and *preventative* were suggested to standardise the advice given to farmers (Vercruysse and Claerebout 2001). The production-based threshold would measure the impact of subclinical infection in the individual or herd, although there is some debate about the threshold fluke burden that would induce measurable clinical changes and lower productivity. Vercruysse (2001) felt that defensible infection intensities that would cause production losses were ≥30 fluke in an individual and / or a herd prevalence of liver fluke infection ≥25%.

Serum gamma-glutamyl transferase (GGT) concentration increased with an artificial infection of 1000 metacercariae to 317 units/L where less than 50 is considered normal, and cattle with 30 flukes having GGT concentrations of 150 units/L (Vercruysse and Claerebout 2001),

Serum GGT is indicative of bile duct damage such as that caused by flukes entering the ducts and feeding, however this enzyme is also elevated with facial eczema damage, making it use problematic in New Zealand. In a study where serum GGT and fluke counts were performed on cattle >24 months of age, as few as 10 liver flukes were determined to impact the individual as determined by an elevated GGT (Charlier *et al.* 2008). The serum GGT concentration was positively correlated to the fluke burden in the cows in February to March (spring) ($R=0.65$; $p<0.001$) after housing in November and December, with the GGT being clearly elevated with >10 flukes present. However, the autumn GGT was less well correlated ($R=0.24$; $p=0.03$) with no significant GGT elevations even when more than 30 flukes were present. This could indicate that migrating flukes cause a greater elevation of serum GGT concentration, and provides a lower benchmark of the number of flukes causing a production-limiting infection after a period of no infection.

A faecal egg count >5epg was also associated with production loss (Malone *et al.* 1990; Vercruysse and Claerebout 2001) which is likely consistent with the low number of flukes required as reported by Charlier *et al* (2008).

In a broader sense it has been noted that the impact at a herd level may not be observed until 25% or more of the herd are infected (Vercruysse and Claerebout 2001; Charlier *et al.* 2007). However, this number does not reflect the level of infection in individual cows, only the percentage of the herd infected and is thus potentially misleading. Both factors could be equally important.

None of these studies include the effect of progressive liver damage that can occur in an individual cattle beast resulting from successive infections over many years.

1.8.1 Growth Rates

There have been many studies reported which show an impact of liver fluke on growth rates of cattle. A meta-analysis (Hayward *et al.* 2021) found statistical support for a 9% decrease in liveweight gain per day due to liver fluke infection. In contrast, in a review it has been reported that the carcass weight in beef cattle infected with liver fluke are reduced by as little as 0.5-0.7% (Charlier *et al.* 2014). In a different study, artificial infections of 600 metacercariae resulting in 54 adult flukes resulting in reduced weight gains by 8% over six months then subsequently had little effect while infection with 1000 metacercariae reduced weight gains by 28% over 6 months (Cawdery. *et al.* 1977). In a separate study in the United Kingdom, cattle with a positive faecal egg count had a 23% reduction in growth rates compared to those with a negative count and treatment with anthelmintics with efficacy against adult, adult plus immature or adult plus immature plus early immature flukes at housing. Growth rates by 71g/day over 112 days with no statistical difference in weight gain between the 3 anthelmintics used (Forbes *et al.* 2015). A clinical trial comparing four anthelmintic treatments in 6-18 month old cattle also resulted in greater weight gain of all the treated groups compared to untreated controls (Elitok *et al.* 2006). Growth rates are impacted more in younger cattle and liveweight impacts increased with time since infection (Hayward *et al.* 2021). A study (Mazeri *et al*. 2017) which included 169,000 cattle processed through a Scottish meat processing plant over two years found 28% had evidence of liver fluke infection detected at slaughter. These cattle overall took

10 days longer to reach slaughter weight than cattle with no evidence of liver fluke infection. As liver fibrosis score increased, so did the days to slaughter. Closer evaluation demonstrated that the presence of 1-10 flukes increased days to slaughter by 31, and more than 10 flukes increased it by 77 compared to cattle with no flukes present. As cattle in this study were 366 - 1,199 days of age at slaughter, this represents increased cost of feeding to the farmer. More studies investigating the impact of small fluke burdens on growth rates of cattle during their lifetime and final carcass characteristics would be valuable to identify the costs of chronic, low grade infection.

1.8.2 Reproduction

Herds with higher antibody titres to liver fluke tend to have longer intercalving intervals (Charlier *et al.* 2007), possibly due to reduced well-being, decreased growth and hormonal changes associated with liver fluke infection (Kaplan 2001). However, these effects are often only detectable when comparing highly positive to negative herds (Mezo *et al.* 2011), indicating that the magnitude of the parasite burden is more important than simply being positive or negative.

1.8.3 Milk Production

In overseas studies on dairy farms with year-round milk production, cows typically enter and leave the herd throughout the study period, which possibly masks any impact of fascioliasis on milk constituents, as these also naturally change during lactation. This situation is very different in New Zealand, where all cows are at a similar stage in lactation due to almost all herds being spring calving. On average milk yield (litres) increases to a peak four to eight weeks after calving followed by a gradual decline to drying off, although the lactation curve of heifers has a lower peak yield but a longer persistence than older cows. Fat yield (kg) and protein yield (kg) also follow a similar pattern to milk yield, increasing in early lactation to then have a steady decline. Fat % and protein % have a reverse standard curve, being high at the start of lactation and then decrease with a nadir that is later than that of peak milk yield, to then increase again through the latter stage of lactation but still ending at a lower level than the start of the lactation. Not all cows fit these models precisely and the shape of the curves is influenced by genetics and environmental factors such as feed quality and quantity. These models provide a good baseline for the expected changes in the milk constituents through the entire lactation in a New Zealand seasonal calving dairy herd.

Many studies (Table 5.1) have shown decreased milk yield (litres) associated with increased antibody titres in bulk milk samples (Charlier *et al.* 2007; Mezo *et al.* 2011; May *et al.* 2020; Takeuchi-Storm *et al.* 2021) and increased faecal egg count (El-Tahawy *et al.* 2017) whilst other studies show no difference (May *et al.* 2019). Very few reliable studies have measured the effects of liver fluke on the different constituents of milk. These are summarised in Table 1.6. Charlier et al (2014) argued that liver fluke infection will decrease milk production by 3-5% while Schweizer *et al.*, when developing a model to determine the cost of liver fluke infection in Swiss alpine milking herds suggested a 10% decrease in milk production resulting from liver fluke infection (Schweizer *et al.* 2005). Charlier *et al.* identified that treatment of infected cows at the end of the previous lactation resulted in a quicker ascent and a higher peak lactation compared to untreated, infected cows (Charlier *et al.* 2012). Similarly, an earlier study showed an 8% increase in milk yield over a lactation (Black and Froyd 1972). In a Swedish study, infected cows that were treated produced an additional 3.1kg milk/cow/day ($p<0.001$) than infected, untreated cows, with uninfected cows producing 2.3kg/cow/day ($p<0.001$)

more than infected, untreated cows (Novobilsky *et al.* 2020). In a different location in Europe a similar response was recorded with herds in Spain which were strongly positive for liver fluke and produced an average of 1.5kg milk/cow/day less than negative herds (Mezo *et al.* 2011). Cow age can also influence the production response with older cows showing a greater reduction of milk production compared to first and second lactation cows (Takeuchi-Storm *et al.* 2021) which could be due to accumulated liver damage. This potential of accumulated liver damage to reduce milk production in the subsequent lactation was shown in a study where cows that had a higher antibody concentration at the end of the previous lactation having a lesser milk production response to treatment (Charlier *et al.* 2012). In this study it was proposed that a correlation between the test reading and intensity of fluke infection resulted in more severe liver pathology which did not resolve sufficiently after successful treatment at dry-off. Of note, significant production losses were reported to occur in herds where the prevalence of liver fluke was 25% or higher (Vercruysse and Claerebout 2001) which supports the determination of this infection prevalence as being likely to impact production.

When developing a model to determine the cost of liver fluke infection in Swiss alpine milking herds, Schweizer *et al*. (2005) reviewed studies and calculated that the presence of liver fluke resulted in a 10% decrease in milk yield while Howell (2015) found that liver fluke caused a 15% decrease in milk yield from high yielding dairy herds with lower effects in other herds. Production impacts are more evident when comparing cows or herds with a high level of infection to those that are negative or have a low level of infection. The impact on milk protein, fat and lactose has been less consistent, with differences noted more between cows or herds with high levels of infection compared to low levels or no infection, with moderate and high levels of infection being similar (Charlier *et al*. 2007; Kostenberger *et al.* 2017; May *et al.* 2020) .

A summary of the results of a number of studies on the impact liver fluke infection on milk production and other health parameters such as somatic cell count and days to first service are shown in Table 1.6.

Table 1.6 Studies investigating the impact of F. hepatica *infection on milk production parameters, in order of most recent study at the top of the table. For the various studies listed where an effect is indicated was shown to be significant (p<0.05) in that study.*

Author and Year	Study details and diagnostic tests used	Study results
Springer 2021	Germany, 596 herds, BME Svanovir	More low BCS cows were positive in regions where seropositivity was high, 1129-1335kg decrease in milk yield in positive cows over a lactation
Takeuchi-Storm, 2021	Sweden, BME IDEXX	Positive herds 580kg lower 305 day ECM in positive cows, 919kg decrease in milk yield for cows in their 3rd lactation or more
Hayward, 2021	Meta analysis on production impacts of liver fluke	No statistically significant impact on milk production (p=0.083), examined four studies
Novobilsky 2020	Sweden, 4 herds over 2 years. Coproantigen and BME Svanovir	Milk yield reduction of 2.3kg/cow/day between infected cows and negative untreated cows. Positive cows that were treated produced 3.1kg /cow/day more than positive, untreated cows
May 2020	North Germany, 1022 and 1318 herds, BME IDEXX	Strong herds had a decreased milk yield of 1.62kg/cow/day compared to negative herds, strong -1.43 less than low herds, strong 0.83 kg/cow/day less than medium. Medium herds produce 0.64 kg/cow/day less than low herds and 0.83 kg/cow/day less than negative herds. Strong herds produced 0.06kg less of both fat and protein /cow/day than negative herds.
Villa-Mancera 2019	Mexico, BME crude ES antigen	Positive herds milk production decreased 0.51-1kg milk/cow/day
May 2019	Germany, 17 herds, FEC	Calving to first service 4.69 days longer in infected cows
Kostenberger 2017	Switzerland, 178 alpine herds, BME Svanovir	Upper quartile ODR had lower annual milk yield of 438kg /cow/year (6%), 2.2% lower fat, 1.3% lower protein compared to negative or slightly positive herds. Anthelmintic treatment increased milk protein next lactation by preventing the decline seen in cows with high antibody titre and not treated, treatment also results in a higher protein content of the milk.
Arenal 2018	Cuba, cross sectional study, 516 herds, BME Svanovir	Herds with ODR >0.6 produced 32% less milk volume than herds with ODR <0.3, herds with ODR 0.3-0.6 produced 18% less
Howell 2015	England, Scotland and Wales, 606 herds, high yielding Holstein-Friesian cows, BME crude ES antigen	15% decrease in milk yield from 25th to 75th percentile herds so impact noted between negative / low infection and highly infected herds
Charlier 2014	Review	Impacts are more than the direct impact on production as infection modulates the immune system response to co-infections.
Charlier 2012	Belgium,12 herds, blind, randomised treatment, controlled trial, monthly BME crude ES antigen, individual milk ELISA every three months	Anthelmintic treatment resulted in a higher peak (1.1kg) and greater persistence (9%) of lactation to gain 303kg in 305-day milk production (3.3%). No effect on fat or protein. First lactation and cows with high result responded well, very high ODR cows did not respond to treatment, either did thin cows (low BCS)
Mezo 2011	Spain, 490 farms, 686 cows, MM3 ELISA milk and serum	High positive herds produced 1.5kg (5%) less milk cow/cow/day compared to negative. High positive cows produced 2kg (6%) less milk/cow/day compared to negative.
Charlier 2007	Belgium, 463 herds, BME crude ES antigen	Herds in the highest 25% of ODR produced 0.7kg/cow/day (3%) milk less than herds in the lowest 25%, also produced less milk fat (0.06%), and had a longer inter-calving interval of 4.7days
Schweizer 2005	Switzerland, modelling	Reviewed studies; decided to use 10% decrease in milk yield due to fluke infection, increased time to first service of 13 days, 0.75 more services per conception as model parameters.
Randell and Bradley 1980		15.4% increase in milk volume after treatment
Ribbeck and Witzel 1979		Infected cows 5-86% decrease in milk volume
Black and Froyd 1972	Treatment every 8 weeks	8% increase in milk volume after treatment

Abbreviations: BCS body condition score, BME bulk milk ELISA, ECM energy corrected milk, FEC faecal egg count, ODR optical density ratio.

1.8.4 Energy corrected milk

Energy corrected milk (ECM) is a standardised measure of production so that you can compare different breeds. It calculates the energy required to produce different components of milk including milk yield, fat and protein. It is a parameter commonly used when assessing factors which affect milk production and uses the following formula (Santschi *et al.,* 2011):

$$\text{ECM (kg/d)} = 12.55 \times \text{fat (kg/d)} + 7.39 \times \text{protein (kg/d)} + 0.2595 \times \text{milk yield (kg/d)}.$$

New Zealand herd test data is recorded as volume and not as weight. The density of milk is estimated at 1.03kg/l and although using volume instead of weight introduces a small bias, this calculation of ECM has been used in other studies (Mason *et al.* 2012).

1.8.5 Other impacts

Sub-clinical infections in sheep are associated with a 20% decrease in packed cell volume and haemoglobin concentration (Sykes *et al.* 1980) with blood loss primarily due to haematophagy of the flukes and parenchymal haemorrhage as a result of migration. Blood can be lost at the rate of 0.2-0.5ml/day/fluke (Dawes 1964) and iron is lost and not reabsorbed. Presumably, similar losses occur in cattle but have not been specifically studied.

Hypoalbuminemia and hyperglobulinaemia occur commonly in the infected host. Hypoalbuminemia is magnified through both blood loss and the liver being the only site of synthesis, but this is worse in sheep than cattle (Anderson *et al.* 1977) and is an example where a protein deficient diet may exacerbate the impacts of liver fluke infection.

1.8.6 Concomitant infections

Liver fluke infection can also have an impact on other organ systems of the body. Cattle co-infected with liver fluke and salmonella had higher fatality rates with lower doses of bacteria, excreted more bacteria for longer and showed a greater extent of tissue infection than animals that were not infected with liver fluke (Aitken *et al.* 1978) while in other studies there has been no interaction between the two infections (Hall *et al.* 1981).

The interaction of concomitant liver fluke infection and bovine tuberculosis (TB) was investigated and experimentally the magnitude of the intra-dermal skin test for tuberculosis was shown to be less in cattle with liver fluke infection in a review (Howell *et al.* 2019). Similarly, in a study of 3,026 dairy herds in England and Wales, there was a lower positivity rate to the skin test in cows that had liver fluke infection at the herd level detected by bulk milk ELISA (Claridge *et al.* 2012) which led the authors to believe that co-infection with liver fluke was resulting in underdiagnosis of bovine tuberculosis. However, in contrast, an Irish study of 1,494 dairy herds found no interaction between the bulk milk ELISA and the TB test. These authors did acknowledge that while there may be an effect, the magnitude was not sufficient to have an impact at the herd level (Byrne *et al.* 2019) although the high prevalence of liver fluke may have biased the results. Bovine TB is still an important animal health concern in regions of the West Coast, requiring annual testing of cows on some farms and subsequent culling animals that test positive.

1.9 Flukicides

The location of the flukes and the method of drug elimination have a significant impact on the concentration of drug to which the parasite is exposed. The route of administration (Martin *et al.* 2009) with oral triclabendazole having a higher efficacy than topical application, and season of treatment where topical application of triclabendazole in the winter was least effective, also impacts the pharmacokinetics of the drug and efficacy of treatment (Sargent *et al.* 2009).

A summary of these active ingredients with flukicide activity are shown in Table 1.7 and products which are registered in New Zealand with flukicidal activity in Table 1.8.

Table 1.7 Efficacy of Drugs to treat Fasciola hepatica in cattle. Adapted from Alvarez et al.(2022)

Flukicide anthelmintic (at recommended dose rate)	Route of administration	Stage of fluke killed	Range of age of fluke killed (efficacy >90%, depending on formulation)
Triclabendazole	Oral	From early immature	1 week
Triclabendazole + Oxfendazole	Oral	From early immature	2 weeks
Triclabendazole	Pour on	From late immature	6-8 weeks
Clorsulon	Injection	Adult	≥12 weeks
Clorsulon	Oral	From late immature	8 weeks
Closantel	Pour on	From late immature	7 weeks
Closantel	Injection	From late immature	7 weeks - ≥12 weeks
Oxyclozanide	Oral	Adult	≥12 weeks
Albendazole	Oral	Adult	≥12 weeks
Nitroxynil	Injection	Adult	≥10 weeks
Nitroxynil + Clorsulon + Ivermectin	Injection	From early immature	2 weeks

1.9.1 Triclabendazole (TCBZ)

TCBZ is a halogenated benzimidazole (BZ) compound with an excellent efficacy against immature and mature stages of liver fluke down to 1 wpi (Boray *et al.* 1983) binding to zone 2 of β-tubulin (Ranjan *et al.* 2017). It is administered orally or as a pour on. After administration the TCBZ metabolites remain in the bloodstream for 144 hours bound to protein (Hennessy *et al.* 1987; Moreno *et al.* 2014) with the metabolites (TCBZ SO and TCBZ SO_2) being orally ingested by the flukes (Lifschitz *et al.* 2017) and target microtubules causing the tegument to strip off (Toner *et al.* 2010b) 48-72 hours (h) post treatment (pt). After treatment egg formation ceases by 48 h with the greatest impact being on the testes. However, it takes some time for flukes to be killed and removed from the liver with flukes recovered from sheep 48 hours post treatment being active while at 72 hours all were dead with a number being present in the gall bladder (Toner *et al.* 2010b), which can confuse some reports on efficacy. In sheep, triclabendazole sulfoxide concentration 24 hours after treatment were 0.41 ug/g in liver, 10.8ug/ml in plasma and 15.2ug/ml in bile (Moreno *et al.* 2014). Detachment of adult flukes

from the bile ducts occurs 48-72h pt with all dead by 96 hours (Toner *et al.* 2010a) and a similar timeline was noted for immature flukes (Halferty *et al.* 2008).

1.9.2 Albendazole (ABZ)

Adult liver fluke (> 12 weeks old) are susceptible to ABZ and its metabolites at 7.5mg/kg in sheep and 10mg/kg in cattle (McKellar and Scott 1990). This is administered orally and has efficacy against flukes including those resistant to TCBZ implying it may bind to a different site on the β-tubulin molecule than TCBZ (Robinson *et al.* 2004; Ranjan *et al.* 2017). ABZ severely disrupt the reproductive system impacting egg development (Lang *et al.* 1980) and viability which can cause stunting of flukes. ABZ has a rapid impact by 48-96 h pi. Fasting of animals to slow rumen transfer may help to increase the absorption and efficacy of ABZ (Lifschitz *et al.* 1997; Sanchez *et al.* 2000) and therefore efficacy against liver flukes.

1.9.3 Closantel (CLOS)

This is a salicylanilide derived compound with high efficacy against flukes >8 wpi and good efficacy in flukes from 6-8 wpi (Mohammed-Ali and Bogan 1987; Lifschitz *et al.* 2017). It is detected in high concentrations in plasma but not bile after oral, subcutaneous injection or pour on application. The flukes orally ingest the plasma protein bound CLOS when ingesting blood and it acts by disrupting energy metabolism in the flukes resulting in severe tegument and gut damage. It has been shown to cause retardation of fluke development from 5 wpi in cattle resulting in an increased prepatent period (Hanna *et al.* 2006). CLOS has a rapid impact on flukes causing death by 24h pt and at 2 days pt only flaccid, inactive and partially fragmented flukes remained in sheep (Hanna 2015).

1.9.4 Nitroxynil (NIT)

A halogenated phenol, NIT as a subcutaneous injection is highly effective against flukes from 8 wpi in sheep (Boray and Happich 1968) and 2 wpi in cattle when combined with clorsulon (Virbac 2020) causing spastic paralysis of fluke. However, the mode of action remains not clear (Fairweather *et al.* 1984). It binds strongly to albumin and persists at high concentrations for up to 10 days in sheep (Moreno *et al.* 2010). NIT has a rapid impact with flukes being dead and autolysed 48 h pt (Hanna 2015).

1.9.5 Clorsulon (CLOR)

A sulfonamide, CLOR can be given as a subcutaneous injection in cattle and an oral drench for sheep and cattle. It targets two glycolytic enzymes causing rapid death of adult flukes from 8wpi within 25-30h pt. It binds to red blood cells with oral ingestion being the main route of entry into flukes (Meaney *et al.* 2004). It may also depress fluke growth and maturation (Malone *et al.* 1984a).

1.9.6 Other compounds

Artemisinins are extracts of the 'wormwood tree' and have shown promising efficacy in rat models but have not proved efficacious in sheep and toxicity to the host is a concern (O'Neill *et al.* 2015).

Mirazid contains a purified oleoresin extract or myrrh from the plant *Commiphora molmol*. To date efficacy studies based on faecal egg count reduction have shown a 83% reduction in sheep (Haridy *et al.* 2003) which indicates that further study would be useful.

Table 1.8 Anthelmintics registered and currently used in New Zealand with label claims of efficacy against liver fluke (2022).

Main active	Actives	Brand names	Application	Species	Milk WHP	Meat WHP
Triclabendazole	Triclabendazole + Oxfendazole	Flukecare + Se	Oral	Cattle, Sheep	35 days, not for use within 28 days of calving	28 days
	Triclabendazole + Abamectin + Levamisole	Switch Fluke 10	Oral	Cattle, sheep	35 days, not for use within 28 days of calving	Cattle 49 days, Sheep 65 days
	Triclabendazole + Abamectin	Genesis Ultra Pour On	Pour on	Cattle	91 days	91 days
	Triclabendazole + Moxidectin	Cydectin plus fluke pour on	Pour On	Cattle	84 days	84 days
Clorsulon	Clorsulon + Ivermectin	Ivacare Plus, Iconix, Icon F, IPlus	Injection	Cattle	14 days	28 days
Nitroxynil	Clorsulon + Nitroxynil + Ivermectin	Nitromec Injection	Injection	Cattle	Not for use in lactating cows or pregnant cattle that may in the future produce milk for human consumption	56 days
Albendazole	Albendazole + Levamisole	Arrest-C, Corporal, TwinAL		Cattle, Sheep	35 days	14 days
Closantel	Closantel + Albendazole + Abamectin + Levamisole	Q-Drench	Oral	Sheep	35 days	28 days
	Closantel + Abamectin + Albendazole	Polerize	Oral	Sheep	56 days	56 days
	Closantel + Abamectin	Genesis Ultra oral, Clomax	Oral	Sheep	56 days	56 days

1.9.7 Flukicide resistance

Anthelmintic resistant fluke are a significant issue, with increasing reports from around the world and New Zealand (Kelley *et al.* 2016; McMahon *et al.* 2016; Fairweather *et al.* 2020; Kelley *et al.* 2020). The failure of fluke treatments will increase welfare and economic costs to farm systems that are reliant on the use of anthelmintics (Sargison and Scott 2011).

Flukicide resistance is separate from the often-limited spectrum of activity of a flukicide against the various stage of the liver fluke life cycle and involves a genetic component that confers an advantage to the population with this genetic code.

Triclabendazole resistance was first detected in Australia in 1995 (Overend and Bowen 1995) and since then has been reported in Britain, Ireland, Netherlands, Spain, Turkey, Peru, Chile, Argentina and New Zealand (Kelley *et al.* 2016) while resistance to other flukicides has also been reported (Table 1.9). Clearly, the number shown in Table 1.9 will be a large underestimate of the actual prevalence of such cases.

Table 1.9 Table of the number of Fasciola hepatica on-farm flukicide resistance cases reported worldwide, adapted from Kelley (2016) and Fairweather (2020).

Flukicide	Reports of on-farm resistance
Triclabendazole	30
Albendazole	3
Rafoxanide	1
Clorsulon	3
Closantel	1
Nitroxynil	1
Nitroxynil + clorsulon	None
Oxyclozanide	None

The methods of detecting flukicide resistance are reviewed by Fairweather (2020). In research settings the use of a controlled efficacy test (CET) involving artificial infection and slaughter of the animals is considered the gold standard but even this has several methodologies, with variations including the sourcing, processing and dose of metacercariae, host species used, time after infection and numbers of animals per group (requiring a minimum of three). In the absence of a standardised methodology, the World Association for the Advancement of Parasitology (WAAVP) guidelines developed for nematodes are often used. A CET is useful to confirm the existence of flukicide resistance but is not practical for investigating at an individual farm level.

The faecal egg count reduction test (FECRT), also without a standardised protocol, has been used to diagnose flukicide resistance in flukes in cattle (Fairweather *et al.* 2020) on individual farms or in small geographical regions. Once again, the WAAVP guidelines for nematodes is used as a guide with successful treatment being efficacy of ≥90-95% but this must take into consideration the registered efficacy of the flukicide. The timing of resampling of animals will depend on the spectrum of activity of efficacy against the various stages of the fluke life cycle for the flukicide being investigated. While the FECRT has limitations which must be acknowledged, it is a useful test when investigating suspected flukicide resistance at a farm level where patent infections are present.

Egg hatch test methodology is a measure of the ovicidal effect of a flukicide. This also has not been well defined in terms of sourcing of the eggs, the time when the eggs are exposed to the flukicide and what duration or the relationship between in vitro results and those seen in vivo (Alvarez *et al.* 2022).

The coproantigen ELISA test detects fluke excretory-secretory antigens (ESA) in the faeces of the host that are reliably detected once flukes are present in the bile ducts. The test has high sensitivity (Mezo *et al.* 2004) and is an alternative to the FECRT partly due to requiring less laboratory time to process samples. The coproantigen ELISA has been used to determine flukicide efficacy in both research settings and in the field (Novobilský *et al.* 2012; Robles-Perez *et al.* 2013; Brockwell *et al.* 2014; Hanna *et al.* 2015; Arifin *et al.* 2016). In the field, the reliability of the test has been questioned, particularly when the host has many immature flukes which may mature before the post-treatment sample is taken (George *et al.* 2017). This may be overcome by sampling the animals one and six weeks post treatment to allow immature flukes to develop and enter the bile ducts (George *et al.* 2019). The coproantigen ELISA does have limitations for determining flukicide efficacy including standardisation of the positive cut-off value, sensitivity in naturally infected animals and lack of validation of the test. At present the coproantigen ELISA is not formally related to numbers of flukes present so an actual efficacy value is not possible to estimate.

While flukicide resistance presents a major threat to the management of liver fluke, the lack of standardisation of testing and inherent weakness of some of the testing options creates diagnostic headaches.

1.10 The Köppen-Geiger system

The Köppen-Geiger system was developed in the late 19th century and recently updated to classify the world into five climate zones and 30 sub-zones to map biome distribution of the world. It is based on criteria such as monthly rainfall and temperature. (Table 1.10). This is relevant when determining zones suitable for the establishment of the intermediate host snails, but consideration needs to be given to small regional variation of climatic conditions which may influence to classification at a local level. The West Coast is classified as *Cfb*; a temperate region, without a dry summer or winter, having a warm summer with four months where air temperature >10°C, but the hottest monthly average is not ≥22°C.

Table 1.10 The Köppen-Geiger system of describing the climatic conditions in a region (adapted from Peel et al 2007). MAT = mean annual temperature, T_{cold} = air temperature of the coldest month (°C), T_{hot} = air temperature of the warmest month (°C), T_{mon10}= number of months with air temperature >10 °C, MAP = mean annual precipitation (mm/y), P_{dry} = precipitation in the driest month (mm/m), P_{sdry} = precipitation in the driest summer month (mm/m), P_{wdry} = precipitation in the driest winter month (mm/m), P_{swet} = precipitation in the wettest summer month (mm/m), P_{wwet} = precipitation in the wettest winter month (mm/m), $P_{threshold}$ = 2xMAT if >70% of precipitation falls in winter, otherwise $P_{threshold}$ = 2xMAT+28 if >70% of precipitation falls in summer, otherwise $P_{threshold}$ = 2xMAT+14. Summer (winter) is the six-month period that is warmer (cooler) between April – September and October – March.

1st	2nd	3rd	Description	Criterion[a]
A			Tropical	Not (B) & $T_{cold} \geq 18$
	f		- Rainforest	$P_{dry} \geq 60$
	m		- Monsoon	Not (Af) & $P_{dry} \geq 100\text{-}MAP/25$
	w		- Savannah	Not (Af) & $P_{dry} < 100\text{-}MAP/25$
B			Arid	$MAP < 10 \times P_{threshold}$
	W		- Desert	$MAP < 5 \times P_{threshold}$
	S		- Steppe	$MAP \geq 5 \times P_{threshold}$
		h	- Hot	$MAT \geq 18$
		k	- Cold	$MAT < 18$
C			Temperate	Not (B) & T_{hot}>10 & $0 < T_{cold} < 18$
	s		- Dry summer	$P_{sdry} < 40$ & $P_{sdry} < P_{wwet}/3$
	w		- Dry winter	$P_{wdry} < P_{swet}/10$
	f		- Without dry season	Not (Cs) or (Cw)
		a	- Hot summer	$T_{hot} \geq 22$
		b	- Warm summer	Not (a) & $T_{mon10} \geq 4$
		c	- Cold summer	Not (a or b) & $1 \leq T_{mon10} < 4$
D			Cold	Not (B) & T_{hot}>10 & $T_{cold} \leq 0$
	s		- Dry summer	$P_{sdry} < 40$ & $P_{sdry} < P_{wwet}/3$
	w		- Dry winter	$P_{wdry} < P_{swet}/10$
	f		- Without dry season	Not (Ds) or (Dw)
		a	- Hot summer	$T_{hot} \geq 22$
		b	- Warm summer	Not (a) & $T_{mon10} \geq 4$
		c	- Cold summer	Not (a, b, or d)
		d	- Very cold winter	Not (a or b) & $T_{cold} < -38$
E			Polar	Not (B) & $T_{hot} \leq 10$
	T		- Tundra	T_{hot}>0
	F		- Frost	$T_{hot} \leq 0$

1.11 Diagnostic Tests

1.11.1 Overview

Clinical fasciolosis is rare in cattle with most disease being sub-clinical without overt pathognomonic clinical signs. This results in likely underdiagnosis of infection. As diagnostic tests have evolved, so has their purpose, moving from simply diagnosing the infection to determining the impact on animal production, particularly weight gain and milk production (Charlier *et al.* 2014).

Some diagnostic tests have developed from documenting gross pathology at post-mortem and identifying the presence of adult flukes +/- scarring of the liver tissue caused by their migration through to non-invasive tests for the detection of parasite eggs, antigen, DNA or fluke specific antibodies. Detection of the parasite eggs in faeces means that a diagnosis can be made in a live animal. Tests that can detect antibodies created by the host against the stages of the liver fluke infection, or fluke antigens have been refined for use with serum, milk and faeces in individuals or pools of samples from several animals. Some quantify the numbers of flukes present or numbers of animals in the pool infected. Antibody tests are an indication of the host's response to the parasite while antigen tests detect the presence of the parasite in serum or faeces. The detection of parasite DNA to increase test sensitivity is a developing field.

When diagnosing infection, it is helpful if the test quantifies the number of flukes present or the percentage of animals in the pool of samples that are infected.

The use of serial testing throughout the year is likely to provide more information than a single test when monitoring herd infection (Sekiya 2013) and using tests in parallel can increase test sensitivity (George *et al.* 2019). However, repeat testing can become expensive and farmers are reluctant to follow this path without good evidence to support its use.

No diagnostic test is perfect, and each has a different role to play in the diagnosis of liver fluke infection. This section provides a description of each test types, application, and limitations.

1.11.2 Gross Anatomy, Liver Scoring and Total Fluke Counting

1.11.3 Gross Anatomy and Liver Scoring

The presence of mature liver flukes in the major bile ducts at post-mortem examination confirms the presence of the parasite. Visual assessment of the liver identifies the parenchymal damage and fibrosis caused by migrating juvenile flukes while the presence of adult flukes results in enlargement and calcification of the bile ducts. Scoring systems have been developed to categorise the degree of pathology present (Table 1.11) (Charlier *et al.* 2008; Martin *et al.* 2009; Sargent *et al.* 2009) .

Table 1.11 Liver Score in cattle to categorise the pathology caused by liver flukes as described by Martin et al. (2009) and Sargent et al. (2009).

Liver Score	Description
Score 0	Absolutely no pathology evident—liver normal colour and consistency with no visible signs of fluke lesions.
Score 1	Small areas of scar tissue and lesions, <5% of liver affected.
Score 2	Moderate areas of scar tissue and lesions, occurring in 5–10% of the liver.
Score 3	Moderate areas of scar tissue, thickening of bile ducts, small to moderate areas of necrosis, pus, 10–20% of liver affected.
Score 4	Moderate to large areas of scar tissue, ''pipe stem'' liver starting to occur. Moderate areas of necrosis, pus, haemorrhage. 20–30% of liver affected.
Score 5	Large areas of scar tissue, and ''pipe stem'' liver. Multiple necrotic foci, pus, haemorrhage, severe degeneration and >30% of the total liver affected

It should be noted that the changes to the physical appearance of the liver are not pathognomonic for the presence of, or previous infection with liver fluke. In New Zealand, the fungal disease pithomycotoxicosis, locally called "facial eczema", due to clinical signs of photosensitisation resulting from liver damage, is a common cause of pathological change to the livers of farmed ruminants (Laven *et al.* 2020) in regions where this fungus grows on pasture. Researchers must be able to identify the differences in pathology of other diseases or at least be aware of the confounding impact of them during examination. Thus, post mortem examination of livers during commercial slaughter is estimated to have a low sensitivity (Se) of only 63-68% although it does have a high specificity (Sp) of 88-100% for detecting the presence of liver flukes (Rapsch *et al.* 2006; Mazeri *et al.* 2016). Liver fluke counts and liver pathology can be determined concurrently with the extent of liver pathology strongly associated to the number of flukes present (Salimi-Bejestani *et al.* 2008).

1.11.4 Total Fluke Count

Precise examination of the liver to identify immature and mature flukes is considered as close to a gold standard of current infection in the animal as possible, with Se and Sp of 99% and 98% respectively (Mazeri *et al.* 2016). The examination can be performed on fresh livers or those stored chilled or frozen. The procedure for removing and counting the flukes involves opening of the gall bladder and major bile ducts, cutting of the liver parenchyma into 1-2cm slices and then soaking in water with the resultant tissues being sieved through a 200um mesh and examined (Wood *et al.* 1995; Charlier *et al.* 2008; Sargent *et al.* 2009). Fluke identification is made easier using a magnifying lens and light source.

Fluke counts are an aggregation of flukes found entire and an accumulation of parts of flukes damaged in the examination process (Anderson *et al.* 1999; Brockwell *et al.* 2013). Identification of oral suckers on fluke parts found and the counting only of these decreases the risk of over estimation of total fluke counts. *Fasciola hepatica* are differentiated from *Fasciola gigantica* based on overall length (3.5cm v 7.5cm respectively) and having more defined "shoulders" (Anderson *et al.* 1999). Determination of immature and mature flukes can be made based on the length of the parasite and the presence of vitelline glands in adults under microscopic examination (Behm and Sangster 1999).

1.11.5 Faecal Egg Counts

Examination of the host faeces for the presence of *F. hepatica* eggs is relatively low cost and most parasitology laboratories will have the equipment to perform the test. The eggs are too dense to float in a saturated solution of NaCl but will float in a saturated $ZnSO_4$ solution although this can cause deformation and damage to the eggs, due to the higher osmotic pressure, interfering with their identification (Crilly and Sargison 2015).

Filtration removes large digesta and involves a weighed faecal sample being rinsed through a tea strainer, parasitological sieves, or the commercial Flukefinder kit (FLUKEFINDER® | Giardia, Trophozoites & Cysts | USA) using tap water. The Flukefinder® is a two-piece tubular apparatus with a coarse mesh in the upper section to prevent the passage of large plant material and a fine mesh in the lower section to prevent the eggs passing through. The lower section is then inverted, and the eggs are flushed into a container.

Sedimentation, where a solution containing the fluke eggs and plant material is transferred into a vertical cylinder and left to stand allowing the transit of eggs down the water column to lay in the sediment. Happich and Boray estimated a fluke egg will sink at a rate of 100 mm per minute (Happich and Boray, 1969) so the time for the eggs to sediment is dependent on the vertical length of the column. The supernatant is decanted using a vacuum before refilling the cylinder with water for further sedimentation and removal of the supernatant. The process is repeated until the solution is sufficiently clear to allow examination of the remaining liquid using a microscope to identify the eggs. This sedimentation process is also referred to as the "Becker system" after the author who described it (Reigate *et al.* (2021)).

The combination of filtration and sedimentation is commonly used for identification and quantification of eggs in faeces. Approximately one third of eggs in the faecal sample are detected (Happich and Boray 1969b) and in some studies, to adjust for this, the reported number of eggs is calculated by multiplying the result by three (Olaogun *et al.* 2022).

The oval shaped, granular, operculated, bile-stained eggs are approximately 90 µm wide and 150µm long being visible through a dissecting microscope at 2X to 4X magnification. The addition of one drop of methylene blue 0.5-1% to the solution before counting stains the plant material blue-green but not the eggs and may aid egg identification. Eggs of *F. hepatica* can be differentiated from those of the rumen fluke, *Calicophoron calicophorum,* due to the latter being colourless (Charlier *et al.* 2008; Crilly and Sargison 2015).

Studies have shown that when used alone, the Flukefinder is more sensitive than the Becker system (Becker *et al.* 2016; Kelley *et al.* 2020; Reigate *et al.* 2021). In some laboratories the two methodologies are used concurrently to improve sensitivity. Faecal egg counts combining both filtration and sedimentation have an estimated low sensitivity ranging from 30% to 88% (Boray *et al.* 1969; Rapsch *et al.* 2006; Charlier *et al.* 2014; Kelley *et al.* 2021b). Increasing the volume of faeces from 4g to 10g increased sensitivity from 42% to 63% (Charlier *et al.* 2008) and Rapsch et al (2006) demonstrated that making repeated counts of 10g faeces from the same sample increased the sensitivity from 69% with one count, to 86% with two and 90% with 3 (Rapsch *et al.* 2006). In herds where 50% are infected increasing the number of animals sampled to 15-20 would be sufficient to

detect infection (Anderson *et al.* 1999). However, there is no correlation between mob FEC and the number of cows shedding eggs (Anderson *et al.* 1999; Novobilsky and Hoglund 2015).

It should be noted that false negative results are more likely in cows with low fluke burdens (Kelley *et al.* 2021b) so the tests are more likely to detect animals with higher fluke burdens. When faecal egg counting is compared to total fluke counts, eggs were detected in only 23% of cows with three or fewer fluke present whereas most cows with 10 or more flukes had a positive FEC using a 10g sample (Mezo *et al.* 2010b). Although increasing the volume of faeces examined will increase test sensitivity, when determining if an animal has a production limiting fluke burden a positive result (any eggs seen) using 4g of faeces was 10.7 times more likely to detect an animal with a heavy infection (> 10 flukes) whereas when 10g faeces was used, a positive result was only 2.5 times more likely to detect a heavily infected animal. The use of a 4g samples is more likely to detect animals with a production limiting infection. Moreover, using 10g of faeces decreased the specificity of the test from 100% to 96% (Charlier *et al.* 2008). The rather low FEC of 5epg in cattle is production limiting (Malone *et al.* 1990; Vercruysse and Claerebout 2001).

The weight of faeces used for FEC analysis varies widely between studies , using 2g (Elliott et al. 2015; Kelley *et al.* 2021; Reigate *et al.* 2021), 3g (Kajugu *et al*. 2015; Novobilsky *et al.* 2016; Calvani et al. *2018; Zafra et al. 2021)*, 4g (Charlier *et al.* 2008), 5g (Mazeri *et al.* 2016; Reigate *et al.* 2021), 6g (Sargent *et al.* 2009) or 10g of faeces (Charlier *et al.* 2008; Brockwell *et al.* 2013; Brockwell *et al.* 2014; Novobilsky *et al.* 2015; Arifin *et al.* 2016; May *et al.* 2019; Ico-Gomez *et al.* 2021).

Additionally, the time since infection impacts egg output of the parasite. After the prepatent period of eight to ten weeks, egg passage increases to a peak at 18-20 weeks post infection (wpi and then decreases to low levels 30-40 wpi (Bouvry and Rau 1986; Boulard *et al.* 1995). It was proposed that calcification of the bile ducts at 23 wpi traps the eggs (Dow *et al.* 1967) and this can result in underestimation of fluke burden. Variation in egg detection in faeces can vary 5-16 fold both between daily, morning and afternoon sampling, with some animals showing greater variation than others. It was also noted that the afternoon samples were both lower in parasite counts and more varied than morning samples (Walker *et al.* 2006; Kelley *et al.* 2021b). Using a rat model, the pre-patent period and fecundity of flukes both decreased as the number of flukes present in the liver increased indicating that faecal egg counting may underestimate high fluke burdens (Valero *et al.* 2006) and conversely overestimate the burden in recent reinfection in low burden situations (Valero *et al.* 2020).

1.11.6 Host antibody response to Excretory Secretory Antigens (ESA)

Experimental studies have shown that cattle recognise and generate antibody responses from two weeks post infection (wpi) to *Fasciola* antigens. The size of these antigens as detected by Western blot are 24, 30-38, 56, 64, and 69 kDa (Santiago and Hillyer 1988). The highest antibody titre can be detected 3-12 wpi and remains high while the parasite persists in the host (Mezo *et al.* 2010a). Most of the major antigens are excretory-secretory (ES) products produced by the liver flukes as a complex mixture of which 73% are proteinases (Walsh *et al.* 2021) and secreted at quite high rates of 0.5-1 µg /adult fluke / hour (Collins *et al.* 2004; Di Maggio *et al.* 2016) . Cathepsin L (CL) make up approximately 45% of these proteinases (Dalton *et al.* 1996; Rojas *et al.* 2014) and are more prominent with adult fluke infections. Five distinct clades of ESA from CL have been identified with clades one, two and five being present in adult flukes and clades three and four only in juveniles (Robinson *et al.* 2008; Cwiklinski *et al.* 2015). These CL proteinases have been shown to aid with parasite virulence (CL1),

tissue penetration (CL2) and suppression of the host immune system. Cathepsin L clades 1A and 1B were determined to be 24kDa in weight (Robinson *et al.* 2008) with diagnostic tests developed that focus on detection of antibodies responding to the clades.

The antibody response of naturally infected cattle, is different to animals that were naïve and then experimentally infected (Walsh *et al.* 2021). Experimentally infected naïve animals have a strong antibody response to CL1 and CL2 whereas naturally infected cattle have a more variable response to CL1 and CL2 and produce a wider antibody response to other antigens including CL5, glutathione S-transferase and a dihydrolipoyl dehydrogenase suggesting that diagnostic tests based on host antibody response to CL1 alone may be unreliable. CL1 can be detected four to seven wpi in experimentally infected animals and usually has a higher titre with adult fluke infections (Kuerpick *et al.* 2013a; Walsh *et al.* 2021).

ELISA tests have been developed to either detect these ESA or the antibodies produced by the host in response to their presence.

1.11.7 Haemagglutination Test

One of the early attempts at a serology test was a haemagglutination test. This initially used a crude mixture of antigens which were subsequently refined after fractionation of the antigens. Subsequently, a useful Haemagglutination (HA) test was successfully developed for cattle by coupling the refined fraction 2 (f2) antigen to sheep red blood cells, increasing the test Se and Sp as well as overcoming the anti-species antibodies issues and used a methodology that required less stringent refrigeration which was important for use outside research facilities (Levieux *et al.* 1992b). The HA test was quicker than current enzyme linked immunosorbent assay (ELISA) at the time (Clonatec, Labsystem, Paris) and was able to be automated. Subsequent to effective treatment of liver fluke in cattle, the HA using the f2 antigen, was negative by 6 months post treatment (Levieux *et al.* 1992b) and was also an effective diagnostic tool in goats (Levieux and Levieux 1994). The exact nature of the f2 antigen has never been revealed but is known to contain a mosaic of antigens including *Fasciola* cathepsins. The HA test has been superseded commercially by antibody and antigen ELISAs.

1.11.8 ELISA tests

ELISA assays may require specialist equipment to detect and quantify liver fluke specific antibodies or antigens, or be available as a cow side test. In the laboratory the colour change is read using an optical reader converting it to an optical density (OD). This sample OD can then be compared to the OD of negative and positive control samples to give a net OD calculation which can be expressed as an OD ratio (ODR) or a percentage of the sample against the positive control (SP%). A qualitative ELISA interpretation can determine positive and negative diagnosis while a quantitative ELISA interpretation determines the concentration of antibodies present and tables can then be used to categorise degrees of infection. ELISA tests can be used for individual animals or groups, using blood, serum, milk, faeces or meat juice.

1.11.8.1 Unrefined Excretory-Secretory Antigen (ESA) ELISA

ELISA tests were developed for use in research settings using gross ESA products obtained from liver flukes harvested from a donor host to detect circulating antibodies in the host. The results were interpreted as positive or negative as determined by a ROC analysis (Salimi-Bejestani *et al.* 2008; Kuerpick *et al.* 2013a) or in quartiles (Charlier *et al.* 2012) with Se ranging from 90-100% and Sp 88% (Salimi-Bejestani *et al.* 2005b; Kuerpick *et al.* 2013a). Salimi-Bejestani et al (2005b) developed an in-house antibody ELISA test using unrefined ESA antigens for the detection of serum antibodies in cattle while at the Liverpool School of Tropical Medicine (LSTM) and evaluated it against a commercial test (Bio-X bovine *F. hepatica* ELISA kit) with *almost perfect agreement* (kappa = 0.82). This LSTM ELISA is the most commonly used antibody ELISA test in published studies (Sekiya 2013). The LTSM has also been the basis of other in-house ELISA techniques for both serum and milk samples (Charlier *et al.* 2008; Kuerpick *et al.* 2012a).

Association between the intensity of liver fluke infection and the serum ESA ELISA has been recorded in some studies (Anderson *et al.* 1999; Charlier *et al.* 2008; Salimi-Bejestani *et al.* 2008) and also when sampling pooled milk samples (Charlier *et al.* 2007).

1.11.8.2 Purified ESA ELISA

The ESA can be purified to improve test characteristics (Cornelissen *et al.* 1999). For example, Mezo et al (2003) fractionated *F. hepatica* ES antigens to improve test sensitivity and specificity in sheep (Mezo 2003; Mezo *et al.* 2007) and cattle (Mezo *et al.* 2010b). The serodiagnostic value of antigens contained in each one of the 4 peaks obtained were assessed with peak IV (range 7-40kDa) being specific for diagnosis of infected animals and is used in the BIO X Diagnostics kits (Bio-X Diagnostics S.A., Rue de la Calestienne, 38 (PAE), 5580 ROCHEFORT, Belgium)

Synthetic production of the ESA epitopes produces a more consistent test and be more readily available, not requiring the extraction and purification of ESA products from live flukes (Levieux *et al.* 1992a; Cornelissen *et al.* 1999).

1.11.8.3 *IDEXX* Fasciolosis Verification Antibody ELISA

The f2 fraction of the ESA is both highly immunogenic and specific for *F. hepatica* (see Section 1.11.7 for its original use) as originally described (Biguet *et al.* 1962) and purified (Tailliez 1970). To isolate this fraction *F. hepatica* sourced from sheep and cattle were washed and freeze dried then using electrophoresis 15 antigenic fractions were identified and numbered 1 to 15. Of these, five fractions were specific to *F. hepatica* of which fractions 1,2 and 7 were considered major. Immunoelectrophoresis using sera from rabbits that were injected weekly with raw *F. hepatica* antigen identified fraction 2 (f2) by the end of the second week of immunisation and characterised as being highly antigenic and specific (Biguet *et al.* 1962).

It was first used in an immuno-haemagglutination assay (Levieux *et al.* 1992b) then developed as a commercially available ELISA previously known as the Pourquier test, now the IDEXX Fasciolosis Verification test to quantify antibodies in serum or milk in either individual animals or as pooled samples. The colour reaction is read as an optical density (OD) at 450nm and the net extinction (NE) for each sample is calculated by subtracting the OD value that develops in the wells containing –Ag

from the OD developing in wells containing +Ag. The kit contains a positive control (PC) and negative control (NC). The sample-to-positive percentage (SP%) is calculated using calculated with the formula:

$$SP\%_{sample} = 100 \times (NE_{sample} / NE_{PC}).$$

Correlation between SP% and levels of infestation for individual sera and pools of sera or tank milk may be interpreted though a scale of crosses ranging from 0 to +++ (Table 1.12).

Table 1.12 Interpretation of the SP% of the IDEXX Fasciola hepatica test in individual or pooled samples (IDEXX Fasciolosis Verification manual)

SP%	**Correlation between test result and infestation level (individual sera)**	**Correlation between test result and the prevalence of infestation within the herd (pools of sera or tank milk)**	**Scale of crosses**
SP% ≤30	Negative for the presence of *Fasciola hepatica* antibodies	No or very weak infestation	0
30 < SP% ≤80	Mild Positive for the presence of *Fasciola hepatica* antibodies	Low infestation (<20% animals are infected)	+
80 < SP% ≤150	Positive for the presence of *Fasciola hepatica* antibodies	Medium infestation (between 20% and 50% of animals are infected)	++
SP% > 150	Strong Positive for the presence of *Fasciola hepatica* antibodies	Strong infestation (>50% of animals are infected)	+++

For individual samples the test result is interpreted as a degree of positivity, whereas for pooled samples the prevalence of infection in the pool can be estimated (see also 1.11.8.6. Bulk milk antibody ELISAs).

Cattle are positive for f2 antibodies from two wpi and reach a maximum SP% at eight wpi with no cross reaction to paramphistome infection (Reichel 2002). However, the test is unable to determine between immature and mature infections or estimate the number of flukes present in naturally infected hosts (Hutchinson 2003). In a study comparing four commercially available ELISA tests in naturally infected cattle identified by faecal egg counts, Munita (2019) found an doubling of the median SP% in untreated cows over 90 days (123 to 283), whereas for those treated with an anthelmintic active against all fluke stages (triclabendazole), the SP% had reduced to approximately 1/5 this value over the same period. This demonstrated the antibodies can be present 90 days after successful treatment. To determine efficacy of treatment, pre and post treatment samples are required to monitor the change of SP%.

A number of studies have reported on the usefulness of this ELISA and shown the sensitivity ranges from 82-99% and specificity 80-99%. However, these values have been noted to vary depending on the season of the test in one study (Table 1.13).

Table 1.13 IDEXX ELISA Sensitivity and Specificity in naturally infected cattle

Author	Reference test used	Sensitivity	Specificity
Hutchinson 2003 serum	NSW Agriculture in-house ELISA	99%	>95%
Charlier 2008 autumn serum	Fluke count	82%	80%
Charlier 2008 spring serum	Fluke count	95%	88%
Rapsch 2006 serum	Bayesian LCM	92%	94%
Molloy 2005 serum	FEC	98.2%	98.3%
Molloy 2005 milk	FEC	97.7%	99.3%

1.11.8.4 *BIO K 211* – Monoscreen Ab ELISA

The commercial BIO X Diagnostics ELISA (BIO K 211 – Monoscreen Ab ELISA *Fasciola hepatica* / indirect, double wells, Bio-X Diagnostics S.A., Rue de la Calestienne, 38 (PAE), 5580 ROCHEFORT, Belgium) is based on the use of the monoclonal antibody MM3 with an affinity for the fraction IV component (CL 1 antigen) of the ES antigens (Mezo *et al.* 2007; Mezo *et al.* 2010b) in both sheep and cattle. The samples and positive control (C+) (control sample provided with the kit) are duplicated in the ELISA plate provided. The optical density (OD) is read using a 450nm filter.

The net optical density of each sample and C+ are calculated by the following formula

Net OD = OD of the Ag coated well – OD of the uncoated well

The Value (Val) for each sample is calculated using the following formula and expressed as a percentage positive (PP) of the C+.

Val = (net OD sample * 100) / net OD C+

The Val % is then interpreted as one of five grades of infestation (Table 1.14) but does not specifically relate to the number of flukes present.

Table 1. 14 Determination of the degree of positivity of the BioX Fasciola hepatica test in individual or pooled samples calculated from the Value % and interpretation of the value % (BIO-X Diagnostics S.A. manual)

Degree of Positivity	**Individual Sample**	**Pool of 10 Samples**	**Test interpreted from Value %**
0	Value % <10%	Value % <5%	No *F. hepatica* infestation
+/-	10≥ Value% <15	10≥ Value% <15	Dubious outcome. Redo the test in a month
+	15≥ Value% <45%	15≥ Value% <45%	Low-grade infestation
++	45≥ Value% <75%	45≥ Value% <75%	Moderate infestation
+++	Value% ≥75%	Value% ≥75%	Heavy infestation

Unlike the IDEXX Fasciolosis Verification test when using pooled samples, the BioX does not attempt to determine the prevalence of infection within the pooled sample but rather categorises the infection as shown in Table 1.14.

When testing vat milk, this test is sensitive enough to detect infection with as few as 12% of the animals sampled being positive. The BIO X test has been reported to have a Se of 99.2% and Sp of 100% in serum and a *almost perfect agreement* between serum and milk samples (kappa = 0.92, p<0.001) (Mezo *et al.* 2010b).

1.11.8.5 Bulk Milk Antibody ELISAs

Bulk milk analysis is a convenient, non-invasive method of detecting antibodies specific to liver fluke. Total IgG concentrations in milk are very high at parturition with colostrum IgG concentrations four times that of serum and decline rapidly during the first week of lactation to be constant at about 5% of the concentration in serum throughout lactation (Farrell *et al.* 2004; Mezo *et al.* 2010a). It must be kept in mind that significant variation in the concentration of antibodies in cows within a herd exists as detected by antibody ELISA (Charlier *et al.* 2012). A good correlation between milk and serum antibody ELISA concentration in cows was noted in several studies (Molloy *et al.* 2005; Reichel *et al.* 2005; Salimi-Bejestani *et al.* 2007; Mezo *et al.* 2010a). Cows with lower levels of infection gave inconsistent results indicating that in herds where infection prevalence is predicted to be low, multiple sampling may give a more reliable indication of infection burden within the herd. In an Irish study, 14 herds submitted bulk tank milk samples four times over a lactation and these were analysed using several different tests: Ildana (recombinant monoclonal CL1); IDEXX Fasciolosis Verification (f2); BioX (monoclonal CL1); and Svanovir (whole ES) antibody tests. All tests demonstrated a similar pattern of change of antibody concentration change through the lactation (Munita 2019). However, the Se and Sp of the Svanovir test was lower than the others at 59% and 96% respectively while all the other tests were 100% and 100% using serum from experimentally infected animals as the gold standard.

In a separate study using paired serum and milk samples from cows, the Liverpool School of Tropical Medicine (LSTM) ESA ELISA had a Se of 96% and Sp of 80% when the serum antibody concentration was used as a gold standard (Salimi-Bejestani *et al.* 2007). The IDEXX ELISA assay had a Se of 92-95% and Sp of 95-99% (Molloy *et al.* 2005; Salimi-Bejestani *et al.* 2007) when tested on bulk milk samples using known infected and infection free cows as the comparison and serum antibody concentration was again used as the reference test. Serum and milk samples from cows analysed with the IDEXX and Euroclone ELISA's were highly associated with kappa values between 0.94 and 0.97 (Duscher *et al.* 2011). These results confirm that milk is a reliable test substrate for detecting antibodies.

A positive correlation between the *Fasciola*-specific bulk-tank milk antibody level and the herd sero-prevalence has been demonstrated. Infection can be detected when in-herd fluke infection prevalence are at least 12.5% using a monoclonal MM3 ELISA (Mezo *et al.* 2010b), 20% using IDEXX ELISA (Sekiya) and 25% using an ES ELISA (Salimi-Bejestani *et al.* 2005a) . Thus these are all sensitive enough to detect a production limiting infection well below the threshold of 25% as suggested by (Vercruysse and Claerebout 2001). Each assay comes with a table to help the user interpret the result when considering the degree of antibody production in the herd at the time of sampling (Tables 1.15 and 1.16).

Table 1.15 Interpretation of the IDEXX bulk milk or pooled serum ELISA (from IDEXX, Fasciola hepatica Antibody Test Kit Instructions)

IDEXX SP% bulk milk	**Interpretation to percentage of herd infected with *F. hepatica***
SP% ≤ 30	No or weak infection
30 < SP% ≤ 80	Less than 20% of the herd infected
80 < SP% ≤ 150	Between 20 and 50% of the herd infected
SP% ≥ 150	More than 50% of the herd infected

Table 1.16 Interpretation of the BioX bulk milk or pooled serum ELISA (from Biox)

Value % calculated from test	**Degree of Positivity interpreted from Value %**	**Interpretation of the degree of positivity**
Value % <10%	0	No *F. hepatica* infection
10≥ Value% <15	+/-	Dubious outcome, retest in one month
15≥ Value% <45%	+	Low-grade infestation
45≥ Value% <75%	++	Moderate infestation
Value% ≥75%	+++	Heavy infestation

1.11.8.6 Application of Antibody ELISA tests

Key questions for the use and interpretation of antibody tests are the dynamics and duration of detectable titres, especially if used to determine the requirement for repeat treatments at intervals. Several studies have investigated this with a variety of different antibody assays.

Fluke specific antibodies can be detected 2-4 wpi (Santiago and Hillyer 1988; Salimi-Bejestani *et al.* 2005b) and remain stable for up to two years under conditions where natural infection is continuing (Ortiz *et al.* 2000). However, it has been noted that ELISA test characteristics can vary between seasons as noted by Charlier *et al.* (2008) (Table 1.17) and this may be due to the immunomodulation effects of different stages of parasite infection or other factors affecting the immune response in the host.

Table 1.17 The variation of test characteristics based on the season the herd was sampled (Charlier et al. 2008)

Season	Unrefined ESA Serum Antibody ELISA		IDEXX Serum Antibody ELISA		Coproantigen ELISA	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
Autumn	94%	84%	82%	80%	98%	90%
Spring	79%	95%	95%	88%	90%	84%

Once the antigen is removed the production of antibodies reduces and this is observed as a declining antibody titre after successful removal of the liver fluke using anthelmintics or self-cure. This key parameter has only been investigated in a few studies and a comprehensive picture remains to be

developed. It is understood that antibody decay is an exponential process thus making a single sample unsuitable for determination of successful treatment or self-cure. Antibody concentration titres also decrease once flukes arrive in the bile ducts (Hanna 1980) and decline after successful treatment of all stages of *F. hepatica*. The former may explain the findings reported by Charlier *et al*. (2008) where they noted seasonal variation. Formal experiments specifically investigating this decline are few. In sheep the antibody concentration declined by about 25% in the four weeks after treatment whereas the coproantigen declined by 90% (Mezo *et al.* 2007). In cattle antibodies can persist at detectable levels for up to six months post treatment (Castro *et al.* 2000). In contrast, Levieux *et al.* (1992) undertook two experiments whilst developing the f2 hemagglutination assay which monitored the decline of titre over time. This assay records the result as hemagglutination units (HAU) which is the lowest serum dilution at which serum is able to hemagglutinate. In the first experiment cows were treated after housing in autumn and serially monitored monthly. These treated cattle showed a steady decline from a titre of about 120HAU down to very low levels by 6 months post treatment. Their titre had effectively declined by over half by 2 months. In the second experiment, naturally infected cattle were housed then treated but only sampled once post treatment. For these cattle the f2 HAU titre has declined nine-fold by 120 days. These two results indicate that there is a rapid decline in antibody synthesis to the f2 antigen after adult worms are removed.

Currently available ELISA tests utilise a variety of different antigens which may show different rates of decline in antibody titre after the flukes are removed from the animals. However, again the number of studies on this aspect are limited and a decline in antibody titre after successful treatment has been shown with some ELISA tests, but not all. In one study (Munita 2019), a number of different ELISA tests were compared when experimentally infected cattle were treated with a panel of different anthelmintics which were effective against either adult or adult and juvenile flukes and resampled once after 90 days. A decline was evident 90 days after treatment for three different mono-antigen tests (IDEXX Fasciolosis Verification ELISA; BIO K 211 – Monoscreen Ab ELISA; Ildana (recombinant CL1)) but not the Svanovir test (E/S protein). However, it is notable that the decline in test results for these three tests were not necessarily to completely undetectable status and it is also notable that the efficacy of these different anthelmintics was not measured by other means, so some treatments may have had some remaining adult flukes.

In another study, experimentally infected cattle were treated with a fully effective flukicide (triclabendazole) and both the coproantigen (see Section 1.11.9.1) and serological titres were measured. The coproantigen titres were negative one week after successful treatment but serum IgG titres had persisted through to the end of the study 28 days later at 70% the OD of positive control animals (Brockwell *et al.* 2013). Similar decreases in IgG have been observed in other studies (Arias *et al.* 2009), even when the treatment was not fully effective. It is notable that such declines are not always observed (Boulard *et al.* 1995). However, the latter study was using an inhouse ELISA where crude excretory secretory material was used as the antigen and this may have influenced the immune response. Other studies have found persistent levels of antibodies in field studies but with questionable efficacy of the anthelmintic used. For example, in a large group of studies to validate the IDEXX ELISA in Australia, antibodies were still present 18 month post treatment but the author concluded this could be due to ineffective treatment as a result of poor drug distribution caused by significant bile duct calcification or anthelmintic resistance (Hutchinson 2003).

Passive antibody transfer occurs in both calves and lambs with antibodies still detected in lambs and calves born to *F. hepatica* infected dams at 11 to 12 weeks of age (Mezo *et al.* 2010a; Novobilsky *et al.* 2014). This gives some indication of decay rate but is poorly representative of the situation with adult cattle. These colostrum derived antibodies would reflect the decay of existing antibodies, not a decline in production and it is in immunologically naïve animals so may not reflect the situation in older cattle. The half-life of IgG1 and IgG2 are approximately 13 and 20 days respectively which would suggest that if antibody production stopped with removal of the adult flukes, then antibody levels should decline to 12.5% of the original titre by 39 and 60 days respectively (Levieux 1990).

Overall, this collection of studies indicate that antibody decline is variable and not easily predictable. This highlights the risk of using a single antibody titre in an animal or pooled group to estimate current infection status.

1.11.9 Antigen ELISA tests.

The sample type collected and time since infection impacts ELISA tests with serum antigens being greater when flukes are migrating through the liver parenchyma and faecal and bile antigens greater when adult parasites are residing in the bile ducts (Sanchez-Andrade *et al.* 2000).

1.11.9.1 Coproantigen ELISA

A commercially available coproantigen test has been available for several years. This test (BIO K 201 – Monoscreen Ag ELISA *Fasciola hepatica* / indirect sandwich, double wells, BioX Diagnostics, Belgium) utilises a monoclonal MM3 antibody assay to detect fluke antigens (Flanagan *et al.* 2011). This test has been developed so it can be used to detect the presence of these antigens in faeces collected from an infected host (Mezo *et al.* 2004). This antigen test is more sensitive at picking up very low fluke burden in sheep than in cattle but still detected 7/7 cows with 2 flukes and 2/7 cows with one fluke (Mezo *et al.* 2004). The lack of reactivity in the other cows with one fluke was thought to be in part due to the flukes being immature and not releasing ES antigens into the gastrointestinal system. In this study, this coproantigen test identified infected cattle at 6 wpi which was 1-5 weeks before faecal egg counts were able to detect these infections.

This commercial test is available as an indirect sandwich with double wells. One row of wells is lined with antibody and the other matching row without. In addition, the kit contains a positive control sample (C+). The optical density (OD) is read using a 450nm filter.

The net optical density of each sample and C+ are calculated by the following formula

Net OD = OD of the Ag coated well – OD of the uncoated well

The Value (Val) for each sample is calculated using the following formula and expressed as a percentage positive (PP) of the C+.

Val = (net OD sample * 100) / net OD C+

Brockwell et al (2013) further optimised this test when they described that by soaking the faeces overnight in the diluent buffer it was possible to increase the OD by 25% without compromising the background OD value and used a cut-off OD of > 0.014 instead of the kit value of 0.15. The use of the

lower cut-off and overnight incubation have subsequently been incorporated into the recognised methodology for this test. The test sensitivity in cattle ranges from 80 - 100% using the manufacturers 0.15 cut-off (Charlier *et al.* 2008; Palmer *et al.* 2014; Kelley *et al.* 2021b) with a specificity of 92 - 100%. Using the lower cut-off of 0.014 the test had a sensitivity of 87-100% and a specificity of >99% (Palmer *et al.* 2014; Kelley *et al.* 2021b).

The assay can detect as few as few as 2 flukes in cattle (Mezo *et al.* 2004) and reliably detect cows infected with 10 or more (Charlier *et al.* 2008) making it a useful screening test in herds.

The actual correlation between coproantigen values and fluke burdens has only been examined a small number of studies. A good correlation of coproantigen value and total fluke count was noted in naïve, artificially infected cattle (R^2 = 0.87; Brockwell *et al.* 2013) at 126 days post infection (dpi). This study involved only 6 cattle with a range of fluke burdens from 15 to 117 but even for this small number did illustrate there was a good relationship. Similarly, in naturally infected cattle, a moderate to good correlation was noted between coproantigen value and fluke burden (Charlier *et al.* 2008; r=0.58-0.69). In the original description of this assay Mezo (2004) demonstrated a good correlation between Val and antigen level which is a good proxy for actual fluke count. Both these later two studies used the originally determined cut-off values as recommended at that time by the manufacturer. Another factor to be considered is the variation from day to day. Brockwell *et al.* (2013) noted there was a variation in Val calculated over time which was weaker and more variable 105-109 dpi than later during infection, reflecting the variable rate of antigen release into the bile and thus the intestinal tract. Similarly, Kelley *et al.* (2021) found that within animal variation in OD value was 2.6 to 8.9 fold which again illustrates the day to day variation in this assay. Although this assay appears to be able to quantify the fluke burden of cattle, no equations were given in these papers to help determine the actual burden of an animal with a specific coproantigen value. The Mezo paper though did provide a graph of the total fluke burden and antigen concentration with a linear relationship as well as a graph of the antigen concentration and OD value also with a linear relationship but did not extend this to actual fluke count.

1.11.10 Polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP)

Detecting parasite DNA in the intermediate host snail or faeces of the host using PCR technology could increase test throughput and decrease cost. Detecting the lifecycle stages in the snail (Rathinasamy *et al.* 2018; Mignaqui *et al.* 2021) could also help to identify high risk pastures. Many PCR techniques have been used to detect liver fluke DNA in faecal samples, targeting the internal transcribed spacer 2 (ITS2) region of the *F. hepatica* genome but the sensitivity of these was low. A technique using loop-mediated isothermal amplification assay (LAMP) has been described (Ai *et al.* 2010) which was 10^4 more sensitive than conventional PCR tests at the time, but these were still less sensitive than other diagnostic tests (Arifin *et al.* 2016; Martinez-Valladares and Rojo-Vazquez 2016). The low sensitivity of these molecular tests could be due to inadequate amounts of amplified DNA, the DNA extraction process, other components of faeces interfering with the assay and the variability of faecal moisture and composition.

The continued preference for molecular testing is likely to see further development of these tests but currently these are still in the development phase.

1.12 Serum biochemistry

Elevated serum gamma-glutamyl transferase (GGT) is an indication of cholestatic liver pathology, linked to ill-health and decreased animal performance (Cuttance *et al.* 2021) although this is also caused by other diseases. The determination of 10 adult flukes being sufficient to cause production loss is based on serum GGT concentrations (Charlier *et al.* 2008) due to fibrosis and biliary interference (Vercruysse and Claerebout 2001). Serum GGT is elevated at 8 wpi in artificially infected cows and more so in the ventral part of the liver compared to the dorsal areas (Wensvoort and Over 1982) but is not seen consistently, possibly due to low levels of infection or other factors (Salem *et al.* 2011; Kowalczyk *et al.* 2018).

Serum GGT and other serum biochemistry tests are indicators of liver pathology caused by liver fluke infection or the results of blood and protein loss but are not specific or pathognomonic. These tests can be useful to the veterinarian to help determine if further investigation of possible liver fluke infection is warranted.

1.13 Lack of Gold Standard

The definition of a gold standard varies between authors, with some requiring 100% Se and Sp. However, common usage is that a gold standard test is the most accurate test available at the time of assessment. In this thesis this common usage will be followed, with the total fluke count referred to as the gold standard test.

There is currently no gold standard test that has sufficiently high sensitivity and specificity when used in the field (Mazeri *et al.* 2016). As discussed in Section 1.11.6 (Host antibody response to Excretory Secretory Antigens (ESA)) the host response to an artificial infection can be quite different to that of naturally infected animals, adding to the complexity.

The total fluke count is considered as being close to a gold standard of current infection (Mazeri *et al.* 2016) but is only useful for research purposes, being too impractical and expensive for use as a diagnostic tool. Often the serum antibody ELISA is used as the gold standard for determining test characteristics, where it should be referred to as the reference test.

The lack of a gold standard test is also problematic resulting in non-perfect tests being used as the basis for assessing a new test. When using an imperfect gold standard test to assess another diagnostic test, the results may either over or underestimate the characteristics of the new test. These limitations can be overcome somewhat using Bayesian latent class analysis and Monte Carlo modelling. The use of Bayesian methods allows for smaller sample sizes and the comparison of multiple diagnostic tests to calculate the Se and Sp of each (Pouillot *et al.* 2002; Rapsch *et al.* 2006; Mazeri *et al.* 2016).

1.14 Summary of Diagnostic Tests

Each diagnostic test has their strengths and weaknesses. Prior knowledge of the management of the population being sampled, the parasite life cycle and the diagnostic question helps to determine which test is best to use. If sampling at a single time point is necessary then using diagnostic tests in parallel

can significantly increase diagnostic sensitivity (George *et al.* 2019), while serial sampling throughout the year (Sekiya 2013) can also provide more diagnostic information.

1.15 Vaccines

To date there is no successful vaccine to protect animals against liver fluke infection and this is in part due to the lack of understanding of the immune evasion mechanisms of the parasite and the host immune response to the parasite (Zhang *et al.* 2021). The ES secretions of the liver flukes regulate the host immune response (Walsh *et al.* 2021). Targets for vaccine development include the cathepsin proteases of the ES complex (Walsh *et al.* 2021), *Fasciola hepatica*-derived molecules (part of the ES complex) (Martinez-Sernandez *et al.* 2017; Ryan *et al.* 2020), glutathione S-transferase (GST) (Stuart *et al.* 2021) and saposin-like protein 1 (SAP_1) (Kueakhai *et al.* 2017).

Given that sheep are not recognised as being able to develop an effective immune response to *F. hepatica* it is difficult to envisage a vaccine that stimulates a natural process within the animal and any effective vaccine will need to use some unique pathway to attack a hidden antigen. For cattle, the life expectancy of flukes is much shorter (Section 2.3.5) but the reason why flukes die over time is not yet determined and may or may not have any involvement of the immune response. Given that flukes ingest blood directly then parallels can be drawn with the approach taken to develop antibody-driven vaccines against some ticks (Guerrero *et al.* 2012) and more recently *Haemonchus contortus (Kebeta et al. 2020)*.

Chapter 2. A comparison of diagnostic tests in naturally infected cattle

2.1 Introduction:

Liver fluke is endemic in some regions of New Zealand (Charleston, 1990, Harris, 1980). However, despite the importance of this parasite the ante mortem diagnosis of liver fluke infection in farmed livestock is still problematic. There are several tests currently available to diagnose liver fluke infection in cattle and these broadly fall into four categories: visual assessment, conventional microscopy, antibody detection and antigen detection. Visual assessment includes liver scoring and detection of flukes in the bile ducts of the liver, conventional microscopy includes total fluke counts and faecal egg counts, with antibody and antigen detection tests using enzyme linked immunosorbent assays (ELISA) which are performed on either serum, milk or faecal samples.

Total fluke count (TFC) is considered the gold standard test for current infection (Mazeri *et al.* (2016)) but is not practical in the field since it can only be completed at postmortem and is also time consuming. Visual assessment of livers for damage consistent with liver fluke, as part of abattoir post-mortem inspections, is a common practice in many countries with the results reported back to the owner of the animals. However, this inspection may report either the presence of adult flukes in the major bile ducts or evidence of liver fibrosis and is at best a cursory way to detect flukes. A confounding factor when determining the cause of liver pathology identified at slaughter in New Zealand is the fungal disease pithomycotoxicosis (facial eczema), a common cause of pathological change to the livers of farmed ruminants resulting in fibrosis and sclerosis (Laven *et al.* 2020). As the purpose of liver inspection is to determine the suitability of the organ for human consumption, not diagnosis of the cause of the pathology, the sensitivity of this diagnostic technique is low (63-68%) although the specificity is higher (88-100%) when detecting the presence of liver flukes (Rapsch *et al.* 2006; Mazeri *et al.* 2016). In the United Kingdom, it was found that the presence of liver pathology was a good indicator of either past or present liver fluke infection, with the extent of liver pathology strongly associated with the number of flukes present (Salimi-Bejestani *et al.* 2008). However, the United Kingdom does not suffer from facial eczema so it is unlikely that these observations would be valid for New Zealand. For this reason, it is important that non-visual methods of liver fluke infection diagnosis are thoroughly researched and validated for New Zealand conditions.

Faecal egg counts (FEC) have historically been used as a relatively simple method of investigating and monitoring disease in sheep and cattle in New Zealand using equipment found in most veterinary clinics. However, the correlation between adult fluke burdens and FEC is not high and also variable (Charlier *et al.* 2008; Mezo *et al.* 2010b). FEC has a poor sensitivity, ranging from 30% to 88% (Boray *et al.* 1969; Rapsch *et al.* 2006; Charlier *et al.* 2014; Kelley *et al.* 2021b) and this low accuracy is further confounded by the lack of a universally accepted protocol for conducting this test resulting in variation between laboratories. For example, the specificity (Sp) of the FEC was found to be higher using 4g of faeces (100%) than 10g of faeces (96%) due to less faecal debris improving the visibility of eggs (Charlier *et al.* 2008).

Several ELISA methods have been developed to detect and quantify liver fluke specific antibodies in the host and have subsequently been released as commercial products. These tests are marketed as being both qualitative and quantitative, whereby a qualitative ELISA result can only determine a positive and negative diagnosis, while a quantitative ELISA can estimate the concentration of antibodies present from which the level of infection can be inferred. ELISA tests can be used for

individual animal samples or pooled samples from a mob. A problem with the antibody based ELISA tests is that after removal of adult flukes with anthelmintic treatment, time taken for a titre to become negative depends on the initial titre and assay used (Munita 2019).

Antibody ELISAs use fluke excretory – secretory antigens (ESA) to coat the wells to detect the presence of host antibodies indicating either current or recent infection. These antigens can only be purified using specialist equipment. The Liverpool School of Tropical Medicine (LSTM) ELISA developed by Salimi-Bejestani (2005b) for detecting bovine fasciolosis, uses unrefined ESA and is the most commonly used ELISA protocol for detecting antibodies in published studies (Sekiya 2013). Further developments involve the use of purified ESA to improve test characteristics, some of which are available as commercial ELISA kits. The IDEXX Fasciolosis Verification test (IDEXX Fasciolosis Verification, IDEXX Europe BV, Hoofdorp, The Netherlands) utilises the f2 fraction of the ESA to detect host antibodies and is both highly immunogenic and specific for *F. hepatica* with sensitivity ranging from 82-99% and specificity 80-99% (Hutchinson 2003; Molloy *et al.* 2005; Rapsch *et al.* 2006; Charlier *et al.* 2008).

The ELISA protocol has also been modified to detect the presence of ESA rather than antibodies in either serum or faeces indicating current or very recent infection. To date, only one of these antigen detection assays has been commercialised, the coproantigen ELISA, BIO K 201 – Monoscreen Ag ELISA *Fasciola hepatica* test (Bio-X Diagnostics S.A. Rue de la Calestienne, 38 (PAE), 5580 ROCHEFORT, Belgium) which detects and quantifies the presence of parasite ESA in cattle faeces (Mezo *et al.* 2004; Mezo *et al.* 2008; Brockwell *et al.* 2013; Mazeri *et al.* 2017) but results can be variable in chronic infections and where fluke numbers are low (Kelley *et al.* 2021b). Again, refinements are ongoing with two recent adjustments to the manufacturer's instructions. These changes include an overnight incubation of the diluted faeces to increase the reactivity of the sample by 25% without compromising the background OD reactivity, and the use of a lower cut-off value to increase the sensitivity of the test (Brockwell *et al.* 2013). Using the overnight incubation and lower cut-off value of 1.4% the test had a reported sensitivity of 87% and specificity >99% (Palmer *et al.* 2014).

The aims of this present study are to investigate and compare diagnostic tools to detect and quantify liver fluke infection in two groups of naturally infected cattle from an endemically infected property in the North Island of New Zealand. The diagnostic tools include visual assessment of livers and total fluke count, conventional microscopic FEC, antibody serum ELISAs and coproantigen ELISA. In particular, the research compared the usefulness of an In-House ELISA, adapted by the PhD candidate, compared to other standard and commercial tests. The ability of each of these diagnostic tests to accurately estimate the true burden of infection was assessed by comparing their results to the gold standard of the total liver fluke count performed at post-mortem.

2.2 Materials and Methods

2.2.1 Animal Selection

The study population were cattle from a beef and sheep farm in north Taranaki, New Zealand (Latitude -38.925 degrees South, Longitude 174.782 degrees East). This farm was selected based on having a history of endemic liver fluke infection and the farmer's willingness to be part of the study. The same farm had previously been identified in 2011 as having triclabendazole resistant *F. hepatica* in sheep (Hassel and Chapman 2012) and liver fluke is commonly detected at necropsies of cull ewes, or at slaughterhouse post-mortem inspections of cattle and sheep. As a result, faecal egg counts are used

to monitor infections, with anthelmintic treatments administered up to twice a year to sheep and young cattle (<3 years old), with older cattle (>3 years old) only receiving annual treatments. The cattle and sheep co-graze pastures on the farm with limited use of forage crops for yearling cattle during the winter. Since identification of the triclabendazole resistance, the farmer has almost exclusively used an oral drench containing abamectin and closantel in sheep and an injectable treatment containing ivermectin and clorsulon in cattle.

Two groups of cattle were selected for the study, 29 culled mixed age beef cows (MAC) and 10 30-month-old finished steers (steers) in the autumn. All animals were born and raised on the farm and were identified with individual ear tags (NAIT tag). The steers were treated with a flukicidal anthelmintic nine months prior to slaughter whilst the cows had received no treatment in the previous 12 months.

2.2.2 Preslaughter Samples

Both groups were blood sampled from the coccygeal vein using an 18 gauge 1” needle and 10ml plain, (red topped) vacutainer. Animals were also faecal sampled on the same day by gently inserting a gloved hand into the rectum, using a fresh glove for each animal, with collected faeces placed in a 70ml plastic pottle, labelled with the animals' visual ear tag. All sampling occurred on farm on 1 May 2017, then serum samples were transported for four and a half hours in an insulated bag containing ice blocks to the laboratory. On arrival at the laboratory, whole blood samples were centrifuged at 1,100 g for 15 minutes (Heraeus Megafuge 40, Thermo Fischer Scientific, Massachusetts, USA) with duplicate serum samples pipetted from each tube and frozen at -20°C. Faecal samples were split with 10g being used for faecal egg counts and the remainder frozen at -20°C for coproantigen analysis.

2.2.3 Liver collection and carcass data.

The cattle were transported three hours from the farm on the day of slaughter to a commercial food abattoir in Eltham, Taranaki. All animals were slaughtered within six hours of arrival where the cattle were weighed pre-slaughter and their carcasses weighed after trimming. On both occasions, cattle were processed sequentially together with lines of cattle from other farms. Cows were slaughtered on 5 May 2017 and steers on 9 June 2017.

Tracking of carcases and the respective offal through the abattoir involved the use of a printed unique sequential identifier number with multiple tear-off replicates. These tear-off portions adhere to the tissues when wet, with only a slight risk of becoming dislodged. Each liver and gall bladder, identified by the unique identifier number, was removed from the processing line by abattoir staff, and placed in a plastic bag. The bagged livers were then packed into a box which contained up to three livers. The livers and gall bladders were then transported two and a half hours by car from the abattoir to Massey University, Palmerston North. It should be noted that the PhD candidate was not permitted to be on the floor of the abattoir and thus relied on abattoir staff for accurate identification and retrieval of all samples.

2.2.4 Liver examination

The livers and gall bladders were initially examined either within three hours of collection (steers) or after chilling overnight (MAC). Each liver was weighed, photographed and scored for visible pathology

using a scoring system developed by Sargent et al. (2009) (Section 3.1.10). After the initial examination of the livers, they were then frozen at -20°C and stored for up to seven weeks.

2.2.5 Fluke Counts

Total fluke counts were undertaken using the frozen livers. The livers were removed from the freezer, placed in high sided plastic trays (to hold the liver and fluids) and thawed over two to four days in the chiller and then held at room temperature. The livers were removed from the plastic bags and cut into segments with sides no longer than 20cm to facilitate handling, then sliced into 1-2cm wide strips. These strips were then massaged and flushed with warm water over a 250µm sieve to remove any flukes with all tissues caught by the sieve. Free tissue caught in the sieve was examined using a 2X magnification illuminated lens to remove suspected partial or complete fluke bodies which were then identified and counted using a stereoscopic dissecting microscope at 4X magnification. Total fluke counts were determined by summing all the entire flukes, all flukes that were ≥75% intact and all segments containing oral suckers. No attempt was made to differentiate adult and immature flukes.

2.2.6 Fluke faecal egg counts

Faecal egg counts (FEC) were performed using a Flukefinder® (Richard Dixon, Idaho, USA) faecal egg detection kit following the manufacturers' instructions with the sedimentation modification described by Reigate (2021; Section 3.1.1). In brief, a tea sieve (mesh size 500µm, 65mm diameter) containing 10g of faeces was placed in a stainless steel bowl with tap water added to cover the faeces to facilitate mixing using a plastic spoon. After mixing, the liquid was collected in the stainless steel bowl which was transferred into the Flukefinder®. Tap water was passed through the Flukefinder® flushing eggs through onto the lower section of the device. Eggs and residual faecal matter from the lower section of the Flukefinder® were flushed into a bowl and then transferred into a 100ml vertical cylinder. Tap water was added to the 100ml mark, allowed to stand for 5 minutes then siphoned down to 5ml before rinsing with an additional 95ml of tap water. This process was repeated three to four times until minimal flocculant remained after siphoning to 5ml. After agitation, the remaining liquid was transferred into a circular counting chamber supplied with the kit. One drop of methylene blue was added and eggs were identified at 4X magnification (Figure 2.1). The total number of eggs counted was divided by 10 to calculate eggs per gram.

2.2.7 Coproantigen ELISA

The *F. hepatica* ESA concentration in the faeces was estimated using a commercial ELISA test (Bio K 201 – Monoscreen AgELISA *Fasciola hepatica*, Bio-X Diagnostics S.A. Rochefort, Belgium; Section 3.1.6). Briefly, rows A, C, E and G of the 96 well plate provided were coated in a polyclonal antibody specific to *F. hepatica* and rows B, D, F and H were coated in a polyclonal antibody not specific to *F. hepatica*. Two grams of faecal material from each animal were individually diluted in 2mLs of the buffer provided and stored overnight at room temperature before centrifuging at 1,000xg for 10 minutes. One hundred microliters of each diluted sample were added into a well in rows A and B, then C/D, E/F or G/H. Wells G1 and H1 are reserved for the positive control (Cpos). The optical density (OD) was read at 450nm using a microplate spectrophotometer (BioTek Epoch 2, Vermont, USA).

The net optical density (Net OD) of each sample and Cpos was calculated by the following formula

$$Net\ OD\ sample\ 1 = OD\ A1 - OD\ B1$$

$$Net\ OD\ sample\ 2 = OD\ A2 - ID\ B2$$

$$Net\ OD\ Cpos = OD\ G1 - OD\ H1$$

The value (Val) for each sample was calculated using the following formula

$$Val = \frac{Net\ OD\ sample}{Net\ OD\ Cpos}\ x\ 100$$

The positive cut-off value was ≥1.4. Coproantigen ELISA was completed for the faecal samples within 26 months of collection.

2.2.8 IDEXX Elisa

The *F. hepatica* specific f2 antibody levels in the serum samples was analysed using a commercial ELISA test (IDEXX Fasciolosis Verification, IDEXX Europe BV, Hoofdorp, The Netherlands) following the manufacturer's instructions and included the supplied positive (Cpos) and negative (Cneg) controls (Section 3.1.9). The IDEXX ELISA was completed on samples within 18 months of collection. The colour reaction was read as an optical density (OD) at 450nm and the net extinction (NE) for each sample is calculated.

$$NE = OD\ \ antigen\,positive - OD\ antigen\,negative$$

The sample-to-positive percentage (SP%) was calculated using calculated with the formula:

$$SP\%\ sample = 100\ x\ (\frac{NE\ sample}{NE\ Cpos})$$

Test interpretation categorises the individual serum samples as; "*negative*" (SP% ≤30), "*mild positive*" (30 < SP% ≤ 80), "*positive*" (80 < SP% < 150) and "*strong positive*" (SP% ≥150) for the presence of *F. hepatica* antibodies.

2.2.9 In-House ELISA

This serum antibody ELISA is based on the method developed by Salimi-Bejestani (Salimi-Bejestani *et al.* 2005b) which became known as the Liverpool School of Tropical Medicine (LSTM) ELISA, with the process fully described in Section 3.1.5. Liver flukes collected from naturally infected sheep domiciled on the same property as the cattle used in this study, were used to obtain ESA as described in detail in Section 3.1.3. Briefly, flukes were washed in warmed PBS (pH 7.6) to remove blood and bile contamination before incubation in Roswell Park Memorial Institute 1640 (RPMI) medium (Thermo Fischer Scientific, Massachusetts, USA) containing 100 U/mL penicillin and 100 U/mL streptomycin overnight at 37°C, 5% CO_2. The supernatant containing excretory secretory antigens (ESA) was collected into 50mL conical tubes and centrifuged at 10,000xg, 4°C for 30 minutes using a fixed-angle rotor. The resulting supernatant was pipetted into 50mL conical tubes, centrifuged at a rigid angle at 10,000xg, 4° C for 30 minutes with the supernatant concentrated using a Vivaspin 20 10kDa centrifugal concentrator (Vivaproducts, Littleton, MA, USA) using a fixed-angle rotor for 70 minutes at 10,000xg, at 4°C. An antiprotease (cOmplete™ Protease Inhibitor Cocktail tablet, Roche, city, country) tablet was added to each 50mL concentrated fraction containing the ESA and stored at -80°C until needed.

Concentrations of protein were assessed using a BCA assay kit (Thermo Fisher, MA, USA). The protein profile and purity were assessed using three concentrations of ESA (32ng, 16ng and 8ng by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Protein dilutions (45ul/well) were prepared using 2x Laemmli sample buffer (Bio-Rad, CA, USA), heated to 95 °C for five minutes before being loaded into a precast gel (Criterion™, 12+2 wells, 4-20% Tris HCl, Bio-Rad) and run at 200V for 50 minutes. The gel was then stained with Coomassie stain, overnight at 4°C before undergoing de-staining with 40% methanol, 10% glacial acetic acid for approximately 5 hours with gentle agitation. The resulting stained gel was then photographed using digital gel documentation system (UVP, California, USA).

The ELISA was optimised at different concentrations of ESA protein using a checkerboard titration of sample dilution and secondary antibody concentration. Briefly, wells of high binding plates (Grenier bio-one 96 well, flat, microlon high binding plates (reference 655061) Germany) were coasted with 100µl of solution containing ESA diluted in 0.05M HCO_3 to a concentration of 2µg/mL, covered and incubated at 4 °C overnight. The plate was washed twice with 300µl of 1x PBS (pH 7.4) /0.05%Tween-20. The wells were blocked with 200µl of 1x PBS (pH 7.4)/0.05% Tween-20/5% skim milk powder (5% blotto) at 37°C for 60 minutes. The 5% blotto was removed by inverting the plate before 100µl of controls or test serum, in duplicate diluted 1:800 in 5% blotto, was added to each well. The plate was covered and incubated at 37°C for 60 minutes. The plate was then rinsed three times before two soaking cycles of 5 minutes with 300µl of 1X PBS/0.05% Tween-20. After washing, 100µl of secondary antibody (HRP conjugated Bovine IgG goat polyclonal, Bethyl Laboratories Inc., MA, USA), diluted 1:60,000 with 5% blotto, was added to each well, covered and incubated at 37°C for 60 minutes. The wells were washed as described above before 100µl of TMB ultrafast solution (Thermo Fischer Scientific) was added per well. Plates were covered and incubated at 37 °C with gentle shaking for 10 minutes. The reaction was stopped with 100ul of 1N H_2SO_4. The optical density (OD) was read at 450nm using a microplate spectrophotometer.

Positive and negative controls were included on each plate. The positive control sample from a naturally infected mixed age cow from the same property was identified as "*strong positive*" (SP% 244) using the IDEXX Fasciolosis Verification test. The negative control sample from a cow born and raised on a farm without any history of liver fluke infection in Culverden, North Canterbury and tested negative (SP% <4) on two occasions using the IDEXX Fasciolosis Verification test.

The mean optical density (OD) for the positive control (Cpos), the negative control (Cneg) and each sample was calculated as the mean of the two OD readings for each, respectively.

$$mean\ optical\ density\ (OD) = (OD\ sample + OD\ duplicate\ )/2$$

The modified OD for the positive control was calculated using the formula

$$modified\ OD\ Cpos = mean\ OD\ Cpos - mean\ OD\ Cneg$$

The modified OD of each sample was calculated using the formula

$$modifed\ OD\ sample = mean\ OD\ sample - mean\ OD\ Cneg$$

The optical density ratio (ODR) was then calculated using the formula

$$ODR = \left(\frac{modified\ OD\ sample}{modified\ OD\ Cpos}\right)$$

with an optimum positive cut-off of ODR≥ 0.42 (Se 67%, Sp 80%) calculated using the serum of 606 cows from 5 herds analysed as part of another study (Chapter 5) in this thesis.

2.2.10 Statistical analysis.

The raw data was summarised by the mean or median and range, categorised by age of cattle, MAC or steers. The distribution of the continuous data was checked for normality using plots and a natural log transformation was applied if the distribution was found to be right-skewed. Initial exploratory data analysis included constructing scatter plots to assess the relationship between two continuous variables and boxplots to assess the relationship between continuous and categorical variables.

The t-test was used to test whether there was a significant difference in the liveweight, IDEXX ELISA SP% values, In-House ELISA ODR values and coproantigen values between MAC and steers. However, for faecal egg counts (eggs/g), number of flukes and liver score data, which were all not normally distributed, the non-parametric Wilcoxon signed rank sum test was used to test whether there was a significant difference between MA cows and steers. For count data, Fisher's Exact Test was used to test whether there was independence between the diagnostic categories of IDEXX, coproantigen and total fluke counts in MA cows and steers.

A series of multivariable linear regression models were built to examine the relationship between two diagnostic tests. Each model was built in a similar method where one diagnostic test was the dependent variable and the second diagnostic test the independent variable. Only two fixed effects were tested in each model, the effect of age and an interaction between age and the independent variable. If a scatter plot of the two diagnostic tests showed a non-linear relationship then a quadratic effect was also tested in the model. All fixed effects tested in the model were retained if $p<0.05$. Prior to building the models, all variables were assessed for normality using a histogram and if necessary $\log_e$ transformed. Model fit was assessed by plotting the residuals and measuring the R^2 value.

All analysis was performed using R studio (RStudio Team (2020). RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/)

2.3 Results

Exploratory data analysis showed that there were two steers with high coproantigen values (54 and 28 respectively) but zero total fluke counts. The blood and faecal samples were collected at the same time by the PhD student, but the livers were collected by abattoir staff. Since a zero-fluke count is incompatible with a high coproantigen result, the two associated livers values were removed from any analyses where total fluke count was a variable.

2.3.1. Liveweights

Overall, the mean liveweight of steers and MAC was 620kg (range 517-686kg). However, the mean liveweight of the ten steers in this study was 100.8kg more than the 29 MAC (95% CI, 55.3 – 146.3kg; t = 4.8, df = 12.5, p-value = 0.0004; Table 2.1).

2.3.2 Liver examination and total fluke counts

The livers were scored for fibrosis (Section 3.1.10) with a median score of 5 for the MAC being significantly higher than the median score of 4 for the steers ($p<0.0001$, Wilcoxon rank sum test). There was no difference of the mean liver weight between the two groups (p=0.92, t. test). Flukes were identified in the livers of 6/8 (75%) steers with median of 3.5 flukes per animal and a maximum of 10 organisms. Flukes were identified in the livers of 28/29 (97%) MAC with a median fluke count of 8 and maximum of 74 organisms. There was no significant difference in the proportion of MAC which had flukes compared to the steers (p=0.12) with the median fluke count being higher in MAC than the steers, (p=0.01, Wilcoxon rank sum test; Table 2.1, Figures 2.1A and 2.2B).

2.3.3 Faecal analysis and coproantigen

Fasciola hepatica eggs were present in the faecal samples collected from 10/10 (100%) steers and 21/29 (66%) MAC. There was no evidence for a difference in median FEC between MAC (0.1 epg) and steers (0.5epg) (p=0.13, Wilcoxon rank sum test). Paramphistome eggs were also visible in some samples (Figure 2.1A).

There is no evidence for a difference in the proportion of animals that were coproantigen positive by ELISA for MAC (25/29 (86%)) compared to steers (5/10 (50%)) (p = 0.09*,* Fisher's exact test*)* or *the* mean coproantigen value between MAC (CV = 19) and steers (CV = 11) (p=0.22, t. test; Figure 2.1B).

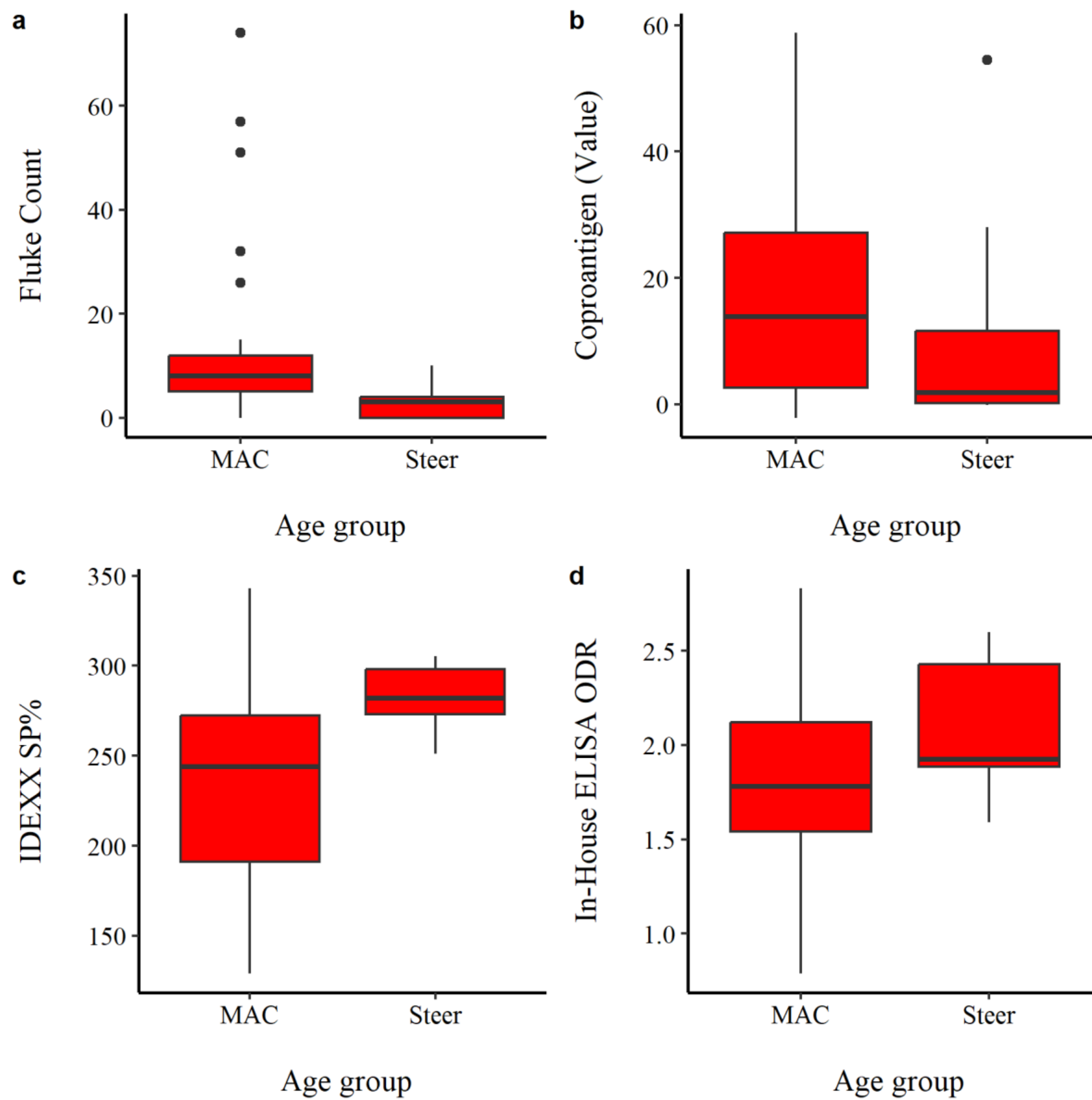

Figure 2.1 A) Box plots of fluke count by age group, B) Box plot of coproantigen value by age group, C) Box plot of IDEXX ELISA SP% by age group, D) Box plots of In-House ELISA ODR by age group.

2.3.4 Antibody ELISAs

In preparation of the ESA protein for the In-House ELISA, 1660ug/mL of crude ESA protein was collected. When protein quality was assessed by SDS-PAGE, bands of protein of molecular weights of approximately 10, 14, and 25kDa were evident on electrophoresis (Figure 2.2 C).

All cattle in both the MAC and steer groups tested positive in the In-House ELISA with positive cut-off ODR ≥0.42. The mean In-House ELISA ODR was 1.9 (range 0.8-2.8) in MAC and 2.1 (range 1.6-2.6) in steers. There was no difference in mean In-House ELISA ODR between MAC and steers (p=0.12, t. test) (Figure 2.1D).

All cattle in both the MAC and steer groups tested positive in the IDEXX ELISA with one MAC being in the positive (80 < SP% < 150) diagnostic category and all others in the strong positive (SP% ≥150) category. The mean IDEXX ELISA SP% was 282 (range SP% 251-305) in steers and 235 (range SP% 129-343) The SP% result for the steers was on average 50 SP% higher than the MAC (95% CI, 25.8– 70.2) SP% (t = 4.4, df = 36.9, p-value < 0.0001) (Figure 2.1C). There was no evidence that the distribution of the IDEXX ELISA category results for MAC was different to steers (p=1, Fisher's exact test).

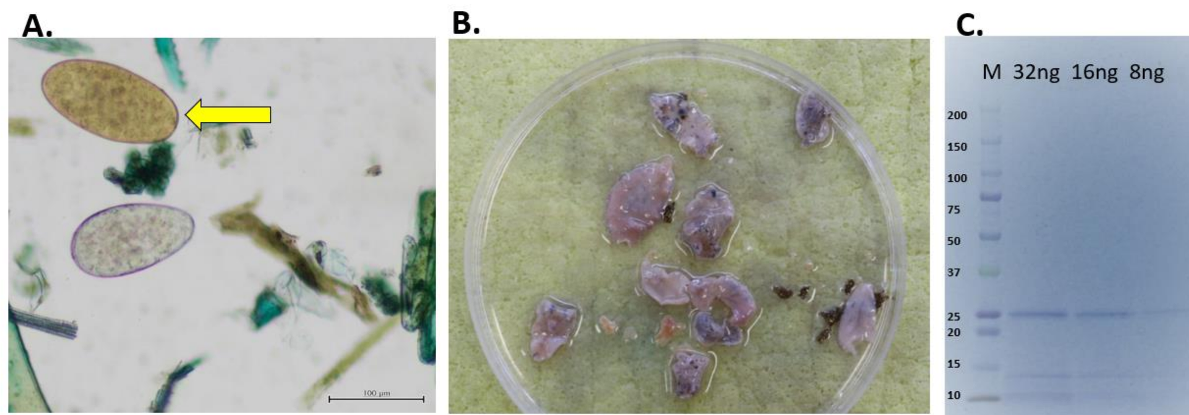

Figure 2. 2 A) Image of a Fasciola hepatica (bile coloured, rounder margins, arrow) and a Calicophoron calicophorum (translucent, smaller) egg under 40x magnification. B) Adult liver flukes and fragments removed during total fluke count and presented on a petri dish. C) SDS-PAGE analysis of ESA at 8, 16 and 32ng concentrations.

Table 2.1 Summary of results of the diagnostics tests performed on samples for the 29 Mixed Age Cows (MAC), 10 Steers and combined over both groups.

	MAC	Steers	All animals
Mean Liveweight kg (range)	520 (416-606)[a]	620 (517-686)[b]	545 (416-686)
Median Liver Score (range)	5 (4-5)[a]	4 (2-5)[b]	5 (2-5)
Mean Liver weight kg (range)	7.6 (6.4-9.1)[a]	7.6 (6.5-8.9)[a]	7.6 (6.4-9.1)
Liver flukes present (%)*	28/29 (97%)[a]	6/8 (75%)[a]	34/37 (92%)
Median number of flukes (range)	8 (0-74)[a]	3.5 (0-10)[b]	7 (0-74)
Median Faecal Egg Count epg (range)	0.1 (0-3.5)[a]	0.5 (0.1-5.2)[a]	0.3 (0-5.2)
Coproantigen positive (%)	24/29 (83%)[a]	5/10 (50%)[a]	30/39 (77%)
Mean coproantigen value (range)	19 (-2.1-59)[a]	11 (-0.1-55)[a]	59 (-2.1-59)
Mean In-House ELISA ODR (range)	1.9 (0.8-2.8)[a]	2.1 (1.6-2.6)[a]	1.9 (0.8-2.8)
In-House ELISA positive (%)	29/29 (100%)[a]	10/10 (100%)[a]	39/39 (100%)
Mean IDEXX Elisa SP% (range)	235 (129-343)[a]	282 (251-305)[b]	247 (129-343)
IDEXX ELISA Category			
Strong Positive (%)	28/29 (97%)	10/10 (100%)	38/39 (97%)
Positive (%)	1/29 (3%)	0/10 (0%)	1/29 (3%)
Mild Positive (%)	0/29(0%)	0/10(0%)	0/39 (0%)
Negative (%)	0/29(0%)	0/10(0%)	0/39 (0%)

[a b] different suffixes in same row denote statistical significance at $p < 0.05$ contrasting MAC and steers

*The total fluke counts for two steers were not included since likely the wrong livers were sampled

2.3.5 Multi-Variable Analysis

2.3.5.1 Association between antibody ELISA tests

To test the association between tests, a multivariable linear regression model was fitted to the data with the IDEXX ELISA as the dependent variable and In-House ELISA and age as the independent variables. The final model showed that there was a significant effect of age on the IDEXX ELISA (p=0.006), with steers on average having an IDEXX ELISA result 48 SP% higher. After adjusting for age there was little support for an effect of the In-House ELISA on the IDEXX ELISA (p=0.06) and no support for an interaction (p=0.46). The residuals from the reduced model were normally distributed with an adjusted R^2 = 0.017. A scatterplot of the data is shown in Figure 2.3

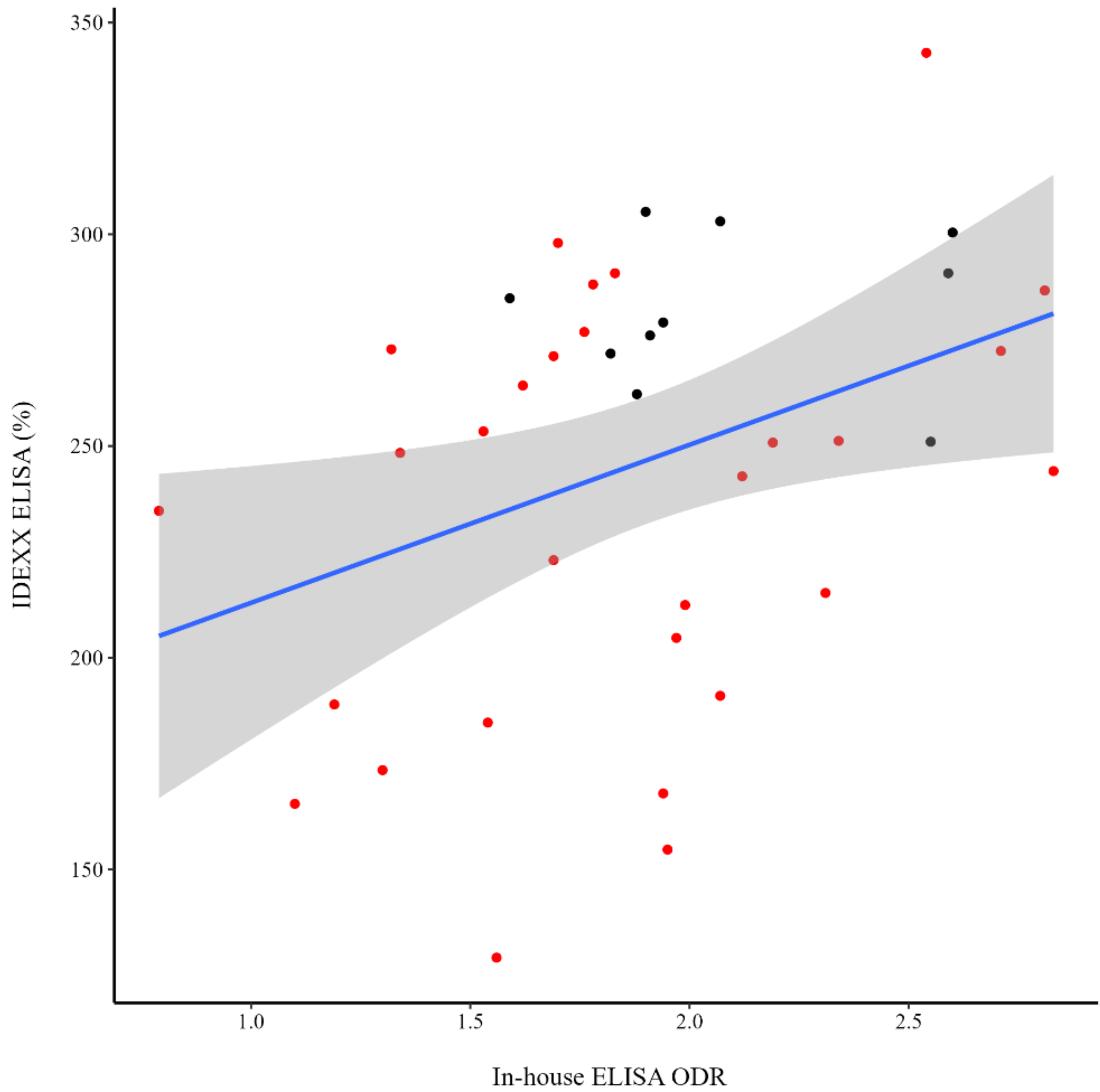

Figure 2.3 Scatterplot of IDEXX ELISA (SP%) against In-house ELISA (ODR) categorised by age, with fitted regression line and standard error. Black dots are steers and red dots are mixed age cows.

2.3.5.2 Association between coproantigen test and total fluke count

A multivariable linear regression model was fitted to the data with the antigen detection coproantigen value as the dependent variable and total fluke count and age as the independent variables. A quadratic transformation of total fluke count and an interaction between total fluke count and age were tested in the model and all variables retained at $p < 0.05$. Total fluke count was first transformed using $\log_e$ (count + 1) due to zero counts and right skewing of the data.

The final model showed a significant quadratic effect for total fluke count (p=0.01). Age was not significant (p=0.28), nor was the interaction significant p=0.54. The final model was:

$$\text{coproantigen value} = -0.96331 + 0.063 * \log_e(\text{fluke count} + 1) + 3.0845 * (\log((\text{fluke count} + 1)^2)$$

Based on the model, the predicted infection of 10 adult fluke would give a coproantigen value of 17.5 (95% CI 13 – 22.1) while a prediction of 30 flukes would have a value of 37 (95% CI 30 – 43.9).

The residuals from the final model were normally distributed with an adjusted R^2 = 0.61. The fit from the model is shown in Figure 2.3 and show a curvilinear relationship between coproantigen results and total fluke count.

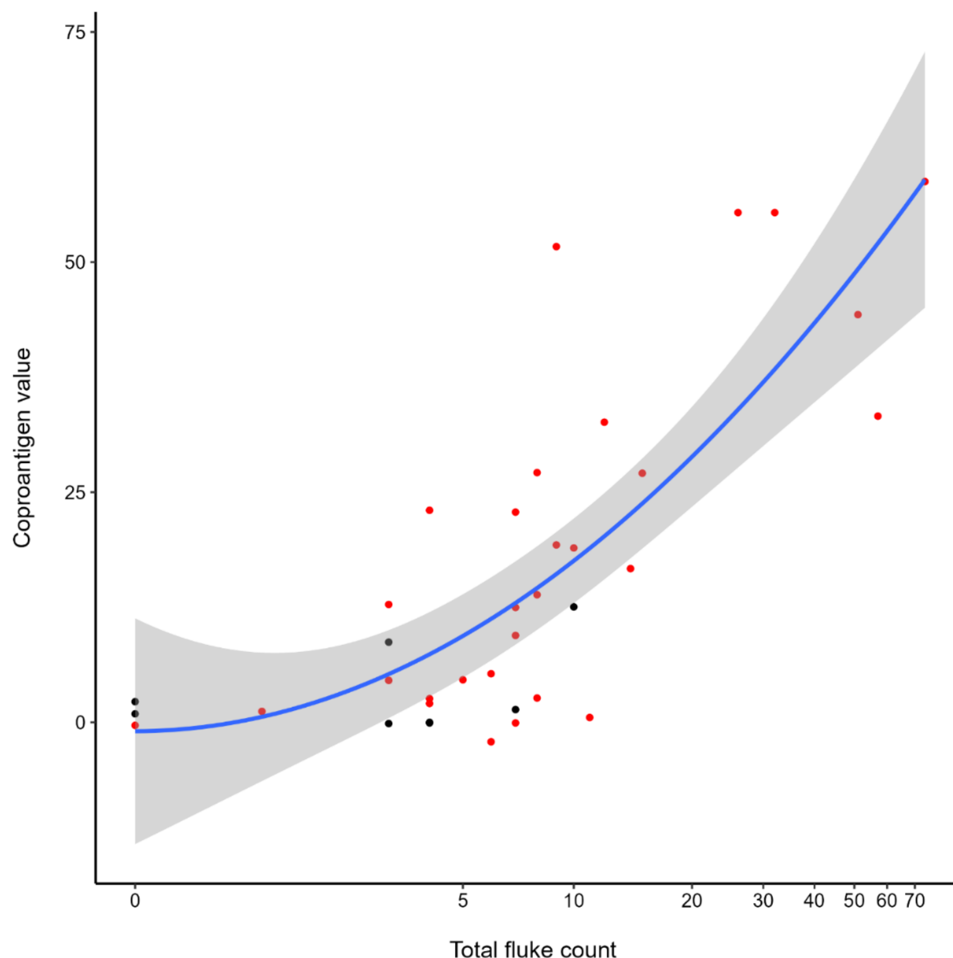

Figure 2.4 Scatterplot of coproantigen value against total fluke count categorised by age, with fitted regression line and standard error. Black dots are steers and red dots are MAC. X-axis is on the natural log scale.

2.3.5.3 Association between coproantigen test and faecal egg count

A multivariable linear regression model was fitted to the data with the antigen detection coproantigen value as the dependent variable and faecal egg count and age as the independent variables. An interaction between FEC and age were tested in the model and all variables retained at $p < 0.05$. Faecal egg count was first transformed using $\log_e$(count + 1) due to zero counts and right skewing of the data. Model fit was assessed by plotting the residuals and measuring the R^2 value. After adjusting for the effect of age (p=0.001), there was a significant effect of fluke egg count on the coproantigen test value (p=0.002). The coproantigen value for the steers was on average 22.5 lower adjusting for the effect of fluke egg count. However, the adjusted R^2 of 0.31 for this model was much lower than for the model using total fluke count as the independent variable.

2.3.5.4 Association between antibody ELISA test and total fluke count

There was no association between the antibody-based ELISA tests and $\log_e$ of total fluke count, IDEXX ELISA (p=0.32) and In-House ELISA (p=0.55). There was no evidence found for an effect of total fluke count on either of the antibody-based tests, IDEXX (p=0.33) and In-House (p=0.58).

2.5.5.5 Association between faecal egg count and total fluke count

There was evidence for an effect of $\log_e$(FEC +1) on log(total fluke count + 1) (p=0.026), adjusting for the effect of age (p=0.01). On average the adult fluke count increased by 1.4 for each unit increase in the log(FEC +1) and controlling for the effect of faecal egg count the steers had 8.72 (95% CI, 3.28 - 14.2) fewer total fluke than the mixed age cows for the same egg count. The adjusted R^2 = 0.23 and the residuals were normally distributed.

2.3.5.6 Association between liveweight and total fluke count

A multivariable linear regression model was built with liveweight as the dependent variable and age and $\log_e$ (total fluke count+1) as the independent variables.

$$\text{lm0} = \text{lm}(\log e(\text{dat\$Total.fluke.Count}+1) \sim \text{age*live.wt.kg,data=dat})$$

The model found a significant effect of $\log_e$(total fluke count+1) (p=0.02) and age (p=0.0004) on liveweight, with no interaction (p=0.13). The model showed that the liveweight fell by 20.4kg for each unit increase in $\log_e$(total fluke count) and steers were on average 79.1kg heavier at slaughter than MAC for the same fluke count, the R^2 = 0.48 (Figure 2.4). There was no evidence found for an effect of FEC on liveweight (p=0.14) or coproantigen value on liveweight (p=0.37)

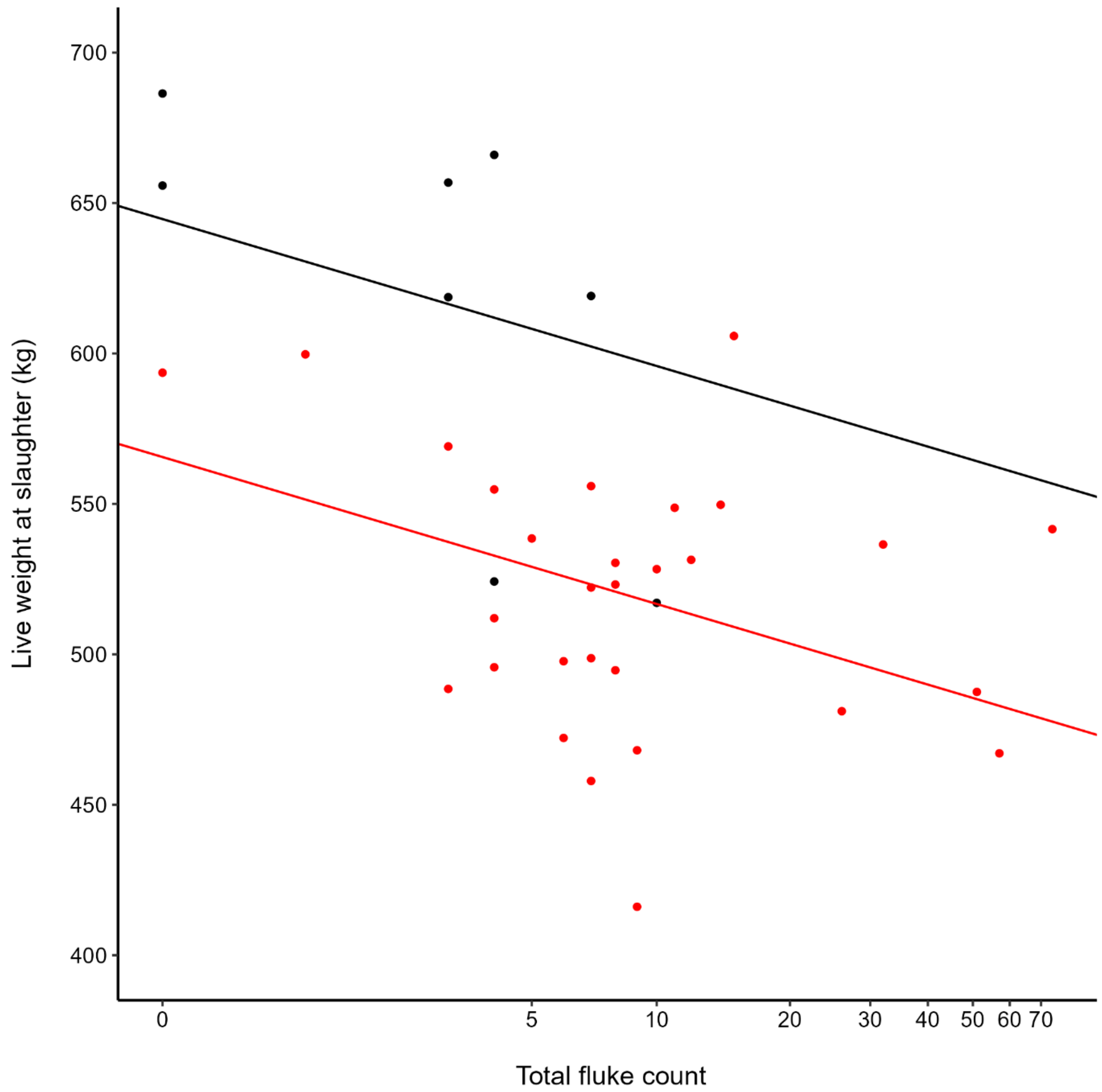

Figure 2.5 Effect of total fluke count on liveweight at slaughter, the red dots are mixed age cattle (MAC) and the black dots are steers, the fitted red line is the regression line for MAC and the black line is the regression line for steers. X-axis is on the natural log scale.

2.4 Discussion:

This study confirms the contradictory nature of liver fluke diagnostic tests in naturally infected animals. In particular, for some of these diagnostic tests, their interpretation without a gold standard is likely to lead to highly inaccurate conclusions. The total fluke count is considered as close to a gold standard diagnostic test as possible for current infection (Mazeri, 2016). In the present study, livers from 37 naturally infected cattle were examined and flukes identified, the practicalities of performing the process on a larger cohort of animals is not to be understated. An added complication studying naturally infected animals is the inability of preslaughter tests to quantify the number of flukes present.

The study had two potential errors, the first was the two steers with high coproantigen values (54 and 28) that had zero total fluke counts, which suggests that mismatched samples or tissues had been collected, and the second was the long storage of faeces before testing (795 days). The slaughterhouse operates as a continuous chain with identification relying on the animals being allocated the correct unique identifier. Many hundreds of animals from different farms are processed in a day, with animals of similar carcass dimensions being processed as a group to improve efficiency of the processing and packaging of the meat and other products, creating the risk of animals being misidentified. Mislabelling may have also occurred between the time of faecal sampling and slaughter although this risk is low as the individual identification of the NAIT tag was available. These two data points were dropped whenever an analysis involved the total fluke count. The faeces were frozen at -20°C for 795 days and in this time the antigens may have degraded reducing the test reactivity. However, this is unlikely in this case as animals in this study had high coproantigen values at the time the samples were tested.

2.4.1 Production limiting infections with liver fluke

In this current study, even though the fluke burdens were relatively low, a linear effect of total fluke count on weight at slaughter was observed with the reduction in liveweight of 20.4kg for each unit increase in $\log_e$(total fluke count). It is important to note that this only reflected the impact of fluke burden at slaughter, not over the lifetime of the cattle. As the study animals, in particular the steers, were sent to slaughter based on the need to decrease the number of animals on farm before the winter period and not selected on weight, live weight is a proxy for weight gain over their lifetime as they were born at a similar time, approximately 30 months prior. Nevertheless, the results from the present study revealed a significant reduction in liveweight which could be related to the fluke burden at slaughter. Our data supports the findings of a Scottish study where naturally infected cattle with as a few as 1-10 flukes took 10 days longer to reach slaughter weight compared to cattle with no evidence of fluke (Mazeri *et al.* 2017). It has to be recognised that the sample of steers (n=10) is small, but this finding is still important and requires further validation.

There have been several attempts to estimate the effect of fluke burden on animal production, at both an individual and herd level (Charlier *et al.* 2008; Vercruysse and Claerebout 2001). Early work involved artificial infection with many hundreds of metacercariae as a single or multiple bolus', where 54 flukes detected at slaughter reduced weight gains by 8-9% in cattle (Cawdery. *et al.* 1977) while 40 and 140 flukes caused 8 and 28% reductions in weight gain respectively (Malone 1986). Reviewing

the literature, Vercruysse (2001) determined that a threshold of ≥30 flukes was a defensible threshold of infection to indicate that treatment would reduce production loss in the individual. However, in adult dairy cattle, Charlier *et al.* (2008) suggested that an infected individual with a total fluke count of >10 adult flukes would cause production losses based on an associated elevation of serum gamma-glutamyl transferase (GGT) in these cattle in the period after housing (spring). The results from the present study indicate a clear relationship between reduced bodyweight and as few as 5 flukes although as indicated above, the fluke burden is measured at slaughter and does not necessarily reflect the burden those cattle had whilst growing. The absence of an interaction by age also indicates that the reduction occurred despite the difference in bodyweight of the two age groups of animals.

Extrapolating the impact of fluke infection in the individual to impact at the herd level are confounded by factors including prevalence of infection and the different stages of infection within the herd at any time point. Significant production losses of lower weight gains and milk production (Koopman 1968) and lower weight gains, food conversion efficiency, milk production and performance of beef suckler operations (Malone 1986) were noted in herds with infection prevalence >25% and this formed the basis of Vercruysse' (2001) defensible position of herd infection prevalence of >25% being a threshold for treatment.

In the present study, the prevalence of infection is very high (92%) in relation to other studies with prevalences of 20-50% (Charlier *et al.* 2008; Salimi-Bejestani *et al.* 2008; Mazeri *et al.* 2016; Zalizar *et al.* 2021) and 78% (Anderson *et al.* 1999). The prevalence in the subset of steers (75%) was similar to other studies for cattle of a similar age (Salimi-Bejestani *et al.* 2008; Mazeri *et al.* 2016). Based on published suggestions for liver fluke burdens, and considered as a whole group, the total fluke counts in this study (median 7, maximum 74) and the number infected (34/37, 92%) are considered high enough to impact animal performance in both the individual; (Charlier et al. 2008) and herd basis (Vercruysse and Claerebout 2001).

2.4.2 Coproantigen as a quantitative diagnostic test

In the present study it was determined that there was a significant association ($p<0.01$) between the antigen-detection coproantigen test and total fluke count with an adjusted $r^2 = 0.61$ which is only slightly less than other studies. Using our sample of cattle, coproantigen test values >17.5 and >37 are consistent with a production limiting infections of 10 or more and 30 or more adult flukes. It was also possible to describe an equation to estimate the actual fluke burden from the coproantigen value which should be of use to others diagnostically. The present study also shows a definite relationship between fluke burdens as diagnosed by coproantigen value and liveweight at slaughter.

An association between coproantigen value and fluke count was determined in naïve, artificially infected cattle in Australia with $r^2 = 0.87$ (Brockwell *et al.* 2013) and in a different Australian study of naturally infected dairy cows with $r^2 = 0.64$ (Kelley *et al.* 2021b). In a Belgian study the coproantigen had a likelihood ratio of 9.7 of detecting cattle with >10 flukes in naturally infected cattle (Charlier *et al.* 2008).

The coproantigen ELISA detects fluke ESA once the flukes are in the biliary system, so the assay is reliant on the ESA protein being present in the faeces and not on the host response to the presence of the flukes. Daily variation of the coproantigen value of 2.6-8.9 fold have been recorded in cattle (Kelley *et al.* 2021b) due to episodic ESA release from the gut of the flukes, faecal consistency

(Brockwell *et al.* 2013), ELISA kit variability (Kelley *et al.* 2021b) and the volume of faeces passed each day. Despite these factors, this study confirms the value of this test to estimate the total adult flukes and detect production limiting infections.

In our study there were some cattle, notably steers, with a positive FEC and negative coproantigen diagnostic category indicating that further studies need to be completed in cattle with chronic, naturally acquired infections of only a few flukes.

2.4.3 Faecal Egg Counts

Previous studies that have monitored *F. hepatica* faecal egg counts have observed a considerable variance between animals with maximum FEC ≥100 epg ((Anderson *et al.* 1999; Phiri *et al.* 2005; Novobilsky and Hoglund 2015), 16-58 epg (Kelley, 2021; Elitok 2006), and counts more similar to this study (FEC<10 epg) (May *et al.* 2019). Although the previous studies also used naturally infected cattle, time since the last anthelmintic treatment was not reported, something that was reported in this study. Studies have shown that the dynamics of egg output after experimental infections reaches a peak 18-20 weeks post-infection (wpi) and then decreases to very low levels 30-40 wpi. (Dixon 1964; Doyle 1971; Kendall *et al.* 1978; de Leon *et al.* 1981). It has been suggested that this shedding dynamic is possibly due to calcification of the bile ducts trapping eggs preventing their transit via bile to the intestinal tract, as the host appears to tolerate the chronic infection and focus on tissue repair (Donnelly *et al.* 2022) with adult cattle usually having low egg counts (Boray 1969). Although it is not known when the cattle used in this study became infected, the farm has a history of chronic infection in both sheep and cattle (Hassel and Chapman 2012). Thus, it is plausible that the infection had become chronic in nature at the time of slaughter in spite of annual anthelmintic treatment. The delay from serum sampling to slaughter (5 days MAC and 28 days steers) was not considered to impact the results, as the cattle had likely been exposed to metacercariae throughout the previous 12 months since last treatment in the cows and seven months in the steers. The farm has a temperate climate, so intermediate host snails are likely to be active from at least spring to autumn ensuring a continuous population of metacercariae over the summer and autumn, but this should be investigated further.

Internationally, an egg count of ≥5epg is considered to indicate a production limiting fluke infection (Malone and Craig 1990; Vercruysse and Claerebout 2001). However, this study showed that reliance on FECs alone could lead to a contradictory result. For example, in this study 28% of cattle had a fluke burden ≥10, indicative of a production limiting infection, however, the FEC were still very low (0.3epg (0-5.2)) inconsistent with a production limiting infection. Charlier *et al.* (2008) reported that the presence of any eggs in 10g of faeces is 2.5 times more likely to indicate an animal with a production limiting infection (> 10 flukes) as compared to an animal with a lower level of infection or not being infected at all. In contrast, those authors reported that the presence of any eggs in 4g of faeces was 10.7 times more likely to detect an animal with a production limiting infection. Therefore, as this current study used 10g of faecal material the sensitivity for the assay could be improved by using only 4g of faeces. Moreover, for sampling cattle in a population where fluke is endemic, the use of 4g of faeces could have more diagnostic value.

2.4.4 Antibody detection ELISA

Taken together, the results of the SDS-PAGE provided confidence that the appropriate ESA proteins had been successfully collected and could be used as the antigen in the development of the In-House ELISA. It should be noted that the analysis of the test characteristics of the In-House ELISA was delayed until further serum samples from other herds were analysed as part of other studies in this thesis (Chapter 4) in order to provide a more robust interpretation. As it was unknown whether in In-House ELISA would be applicable to this study, the commercial IDEXX antibody detection ELISA was also used and results compared.

All the animals tested in this study were positive in both serum antibody ELISA test, with 38/39 (97%) IDEXX results being *strong positive*, and all 39/39 In-House ELISA ODR results at least twice the value of the positive cut point of 0.42(mean 1.9), even in animals with no fluke present. Moreover, many of the positive sample readings were beyond the linear range of the assay. Antibody concentration in the host is dependent on both parasite and host factors and has been shown to be a complex interaction in naturally infected animals with repeated and chronic liver fluke infection (Gorman *et al.* 1997; Ortiz *et al.* 2013; Walsh *et al.* 2021). The very high antibody ELISA results made test comparisons difficult and as a result, further studies are required in populations with a greater range of antibody concentrations to determine if there is any association between anti-fluke antibody titre and current fluke burdens.

Furthermore, identified limitations of antibody ELISA assays include the inability to distinguish between current and past infection, seasonal impacts on Se and Sp and inability to quantify current infection.

As these antibody assays detect the host response to the presence of the liver flukes, they report past exposure. The antibody titre detected in serum or milk could be due to new infection, the continued development of the fluke life cycle within the host, fluctuations of the hosts immune response or antibody decay from an initial high concentration (Mazeri *et al.* 2016) with the difficulty for the veterinarian being that none of these potential reasons are easily identified.

Seasonal variation of Se and Sp has been identified in studies using the IDEXX and ESA ELISAs and was considered in part to be due to the temporality of metacercariae on pasture and the time of sampling. In an Irish study, Se was higher during winter compared to summer and autumn (0.94 and 0.72 and 0.82) with Sp higher in winter compared to summer and autumn (0.89 v 0.87 and 0.76) while in Belgium the spring sampling Se was higher than autumn (0.95 and 0.82) as was Sp (0.88 and 0.80) (Charlier *et al.* 2008; Mazeri *et al.* 2016). These are considerations a veterinarian should consider when interpreting results as host antibody response is greatest 3-12 weeks post infection (Mezo *et al.* 2010a) although the IDEXX SP% can double over 90 days as the fluke infection matures and without further infection (Mazeri *et al.* 2016). The inability to quantify the current fluke burden is a frustration identified in studies including assessing the value of f2 antibody detection assay in Australia and Belgium (Hutchinson 2003; Charlier *et al.* 2008) but in another abattoir study in the UK where liver fluke intensity was determined by a single cut to the ventral liver and then visual pathology scoring, a relationship was found between the LSTM ELISA and intensity of infection (Salimi-Bejestani *et al.* 2008).

In addition, sample quality, storage, storage container and repeated thawing and refreezing of samples should be considered as these factors can affect antibody concentration and aggregate

formation which impact reactivity in ELISA assays (Kueltzo *et al.* 2008). Although some samples were stored for a long period, the use of replicated samples in the present study limited the number of freeze/thaw cycles. It can become an issue for positive and negative control samples. In the present study these were also split, and the number of freeze/thaw cycles was limited.

2.4.5 Summary

Taken together, the results from this study revealed that the coproantigen ELISA was superior to other diagnostic tests at quantifying the fluke burden in naturally infected cattle. The presence of one egg in a faecal sample confirms infection while the antibody ELISA tests reflect the immune response of the host, but in this study the coproantigen gives the veterinarian the ability to determine the number of flukes present and production cost of that burden as indicated by the lower slaughter weight of animals with higher fluke counts.

The In-House antibody ELISA does not appear to be satisfactory for use in further studies so will be replaced with the commercial IDEXX ELISA for further analysis of fluke infection and impacts on milk production. The coproantigen ELISA is the test of choice for determining if cattle are infected with sufficient liver flukes that treatment would improve production.

2.5 Ethics permission

This study was performed under the approval of Massey University Animal Ethics Committee, approval MUAEC 15/92

2.6 Supplementary Materials

Supplementary materials are contained in Section 3.2.1.

Chapter 3: The prevalence of liver fluke infection on farms supplying milk to on Westland Milk Co-operative detected by bulk milk antibody ELISA in the autumn and spring and a short survey of farmer awareness and management of liver fluke.

3.1 Introduction:

Fasciolosis, caused by the trematode parasite *Fasciola hepatica* (liver fluke) is a worldwide problem (Vercruysse and Claerebout 2001; Pritchard *et al.* 2005; Charlier *et al.* 2014; Kelley *et al.* 2020; Waal. and Mehmood 2021) resulting in economic losses for farmed ruminants which is considered to occur where the in-herd prevalence of infection is 25% or more. This "production-based threshold" is described as the level of infection and sub-clinical parasitism that will cause measurable impacts on hosts resulting in a decrease in production including weight gain and milk volume (Vercruysse and Claerebout 2001; Charlier *et al.* 2007). In this thesis, an in-herd prevalence of infection ≥20% as indicated by the diagnostic categories of the IIDEXX Fasciolosis Verification Test of *medium* and / or *strong infestation* are referred to as "production limiting". Studies of liver fluke in New Zealand are generally sparse (Harris and Charleston 1980; Charleston *et al.* 1990; Haydock 2016) with the parasite being more important in some areas than others. The West Coast of the South Island is historically recognised as a region with a high prevalence of liver fluke infection (Charleston *et al.* 1990).

A population of the intermediate host snail is critical for the maintenance of liver fluke infection on farms and in regions. In New Zealand, two species of such snails are found, *Pseudosuccinea columella* and *Austropeplea tomentosa,* with *P. columella* now being considered the most important. First detected in New Zealand in 1940, *P. columella* was thought to have been accidentally introduced into New Zealand from North America and has now progressively replaced the indigenous species *A. tomentosa* (Harris and Charleston 1980; Charleston 1997). Although it is believed that *P. columella* is present on the West Coast of the South Island, the most recent detailed study delimitating the spatial distribution of suitable intermediate host snails was in the early 1970s and at that time it was not found there (Harris 1974a).

These mud snails prefer habitats where their feed source of benthic algae grow; shallow water, muddy substratum and limited shading. They are most active at temperatures between 12°C and 30°C, which closely agrees with that required for successful development of liver fluke intermediate stages. (Pullan *et al.* 1972). The West Coast is noted for frequent rain and flooding events on both a paddock and river valley scale which helps to provide both a suitable habitat for the snails as well as suitable conditions to distribute the snails, parasite eggs and metacercariae to neighbouring farms and areas.

Systematic testing for the presence of liver fluke in New Zealand cattle is sporadic. Testing may include post-mortem liver inspections at slaughter, which can either report the specific presence of liver flukes or the less specific presence of liver damage, with the potential for the latter to be confused with sporidesmin toxicity (facial eczema), which is widespread throughout much of the North Island (Cuttance *et al.* 2021). Slaughterhouse based data is also compounded by the movement of livestock to different regions during their lifespan resulting in liver fluke infected animals being identified in

regions where the parasite is not endemic. Serum and faecal sampling may also be carried out at both the individual animal and herd level, for example during an ill-thrift investigation, a veterinarian may sample individual animals, whereas the bulk milk ELISA (BME) test offers a convenient method to monitor disease in lactating dairy cows at the herd level.

Treatment of liver fluke requires specific anthelmintics, which often have limited efficacy against other parasites. These products have milk withdrawal periods ranging from 14 to 91 days or may not be registered for use in lactating cattle where the milk is intended for human consumption. As a result of these legal restrictions, dairy farmers in regions with a history of liver fluke infection tend to treat dairy cattle at the end of lactation and possibly again during the non-lactating (dry) period. Unfortunately, this creates a mismatch between choosing the optimum time to treat to disrupt the life cycle of liver fluke and choosing the pragmatic time when they can treat to avoid supplying contaminated milk for human consumption. For calves and heifers, these restrictions do not apply and since these cattle are treated more regularly for gastrointestinal worms, then liver fluke treatments can more easily be incorporated into these programmes.

Westland Milk Co-operative (now Westland Milk Products) is the sole milk processor on the West Coast of the South Island and as such is the destination for most milk produced on the West Coast as well as for a cluster of farms in Canterbury.

The West Coast of the South Island of New Zealand lies between latitude 41 to 43 degrees South and latitude 169 to 172 degrees East. It is boarded to the west by the Tasman Sea and east by the Southern Alps with 1600-4000mm annual rainfall and mean month temperature ranging from 5 to 18°C, being classified by the Köppen-Geiger climate zone classification system as Cfb (temperate, no dry season, warm summer). Spring calving dairy cows are pasture fed, with a non-lactating period of around three months over the winter, with cull cows removed from the herd at the end of lactation and heifers being introduced. Liver fluke infection is endemic in many parts of the West Coast and anthelmintic treatment targeting liver fluke may be used in the non-lactating period. Areas of the West Coast with pakihi wetlands, characterised as infertile with little or no peat and poorly drained (landcareresearch.co.nz)) have been developed into farmland through drainage remediation (Thomas *et al.* 2007) with one common method being "humping and hollowing". Using mechanical diggers, soil is dug to form "hollows" and placed in linear "humps" (Figure 3.1) with the resulting hollows being either permanently or predominantly wet areas, creating an ideal environment for mud snail intermediate hosts of *F. hepatica*. Flooding events can also carry metacercaria to neighbouring farms.

By contrast, the province of Canterbury lies at a similar latitude to the West Coast but further east on the opposite side of the Southern Alps mountain range and boarded by the Pacific Ocean to the east. Although given a similar Köppen-Geiger classification to the West Coast, Canterbury is drier with 500-800 mm annual rainfall and the summers are generally hot with long dry periods. The soils are typically free-draining light top soils over alluvial gravel and central pivot irrigation is a common feature of dairy farms throughout Canterbury. Cows calve in the spring with pasture being the dominant feed during lactation and lactation ends in the late autumn or early winter. During the dry period, brassicas, bulb crops and silage are commonly fed. Liver fluke infection is not considered to be endemic in Canterbury, indeed considered to be uncommon.

The aim of this study is to assess the prevalence, intensity, and spatial distribution of *F. hepatica* infection in dairy herds in Canterbury and the West Coast supplying milk to Westland Milk Co-

operative in the autumn and spring of 2017. This study also measures the change in infection status of herds from the end of one lactation to the start of the next and assesses the repeatability of the bulk milk ELISA test. In addition, the results of a short postal survey to determine the awareness of *F. hepatica* infection on dairy farms on the West Coast and the anthelmintic practices used by these farmers to control liver fluke are presented.

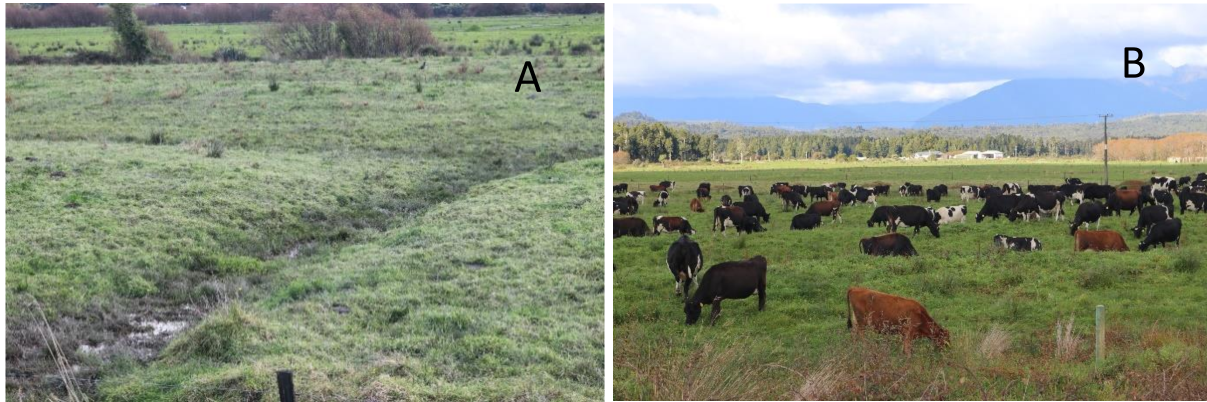

Figure 3.1 The common West Coast technique of "Humping and Hollowing" of farmland to permit drainage and increase the grazable area of the farm. The hollows remain wet and are a suitable habitat for the intermediate host snails of F. hepatica B) Cattle grazing all of the paddock including the hollows.

3.2 Materials and Methods:

3.2.1 Study population and area

This was a cross-sectional observational study and the sample population was all the dairy herds that supplied milk to Westland Milk Co-operative (now Westland Milk Products) over the period of the sampling. Each herd was identified by its unique Westland Milk Co-operative supplier number, which was linked to the region the herd was from and its GPS coordinates (latitude and longitude). Most farms supplying Westland Milk Co-operative are on the West Coast ranging from Karamea and Murchison in the north to Fox in the south. A cluster of farms in Canterbury that also supply milk to Westland Milk Products are also included.

3.2.2 Milk sample collection

A 50-70mL milk sample, identified by the unique farm Westland Milk Co-operative supplier number, was collected from the bulk milk tank of each herd. The milk samples were collected over two consecutive days to ensure maximum enrolment of herds. In March 2017 (autumn), near the end of lactation, milk samples were collected over 2 days on two separate occasions, one week apart and in October 2017 (spring) near the beginning of the subsequent lactation milk samples were collected from a single two-day period. The samples were stored in a refrigerator on the day of collection and transported with cool packs to Massey University, Palmerston North, where they were held at 4°C overnight or stored at -20°C for no more than 5 days then thawed at 4°C for prior to analysis.

3.2.3 ELISA

The bulk milk ELISA (BME) antibody titre was determined by analysing the *F. hepatica* specific f2 antibody concentration in the bulk tank milk (BTM) milk samples using a commercial ELISA test (IDEXX Fasciolosis Verification, IDEXX Europe BV, Hoofdorp, The Netherlands) following the manufacturer's instructions (Section 3.1.9). The colour reaction is read as an optical density (OD) at 450nm and the net extinction (NE) for each sample is calculated by subtracting the OD value that develops in the wells containing –Ag from the OD developing in wells containing +Ag. The kit contains a positive control (PC) and negative control (NC). The sample-to-positive percentage (SP%) is calculated with the formula:

$$SP\%_{sample} = 100 \times (NE_{sample} / NE_{PC}).$$

Test interpretation categorises and describes the prevalence of herd infection level based on the SP% (Table 3.1).

Table 3.1 Bulk milk SP%, diagnostic category and predicted infection prevalence of the sampled group as described in the IDEXX antibody ELISA test instructions.

IDEXX SP%	SP% ≤30	30 < SP% ≤ 80	80 < SP% < 150	SP% ≥150
Infestation diagnostic category	*no or very weak*	*low*	*Medium*	*strong*
Predicted sampled group infection prevalence		<20%	≥20%	>50%

3.2.4 Repeatability of the IDEXX F. hepatica ELISA

To determine the repeatability of using the IDEXX ELISA to categorise the herd prevalence of liver fluke infection in a dairy herd, samples from 99 herds were analysed in both weeks in March 2017. Samples from herds submitted in the second week of collection were eligible if a sample had also been analysed in the first week. These eligible milk samples were placed in a plastic bag and 99 were randomly selected by drawing from the bag.

3.2.5 Survey questionnaire

A questionnaire (Appendix 3) was included with the IDEXX *F. hepatica* ELISA result letter sent in May 2017 to all farmers whose herd BTM was sampled and analysed using the BME in March 2017. The survey questionnaire was evaluated by peer review and judged to be low risk and approved by the Massey University Human Ethics Committee. A cover note accompanied the postal survey, describing its purpose, assuring anonymity, and identifying the researchers with their contact details. Farmers were asked to voluntarily supply their milk supplier number, and this allowed the questionnaire to be linked to the BME test result. Where a farmer owned more than one herd and gave the milk supplier numbers for those herds, these questionnaire results were recorded against each unique supply number e.g., if a farmer supplied three milk supplier numbers, then the results were entered as three individual herds.

The survey contained 6 closed questions with tick boxes next to the available answers. The respondents were asked about,

1. Their awareness of liver fluke presence on their farm in the previous five years,
2. Did they drench the herd specifically targeting liver fluke,
3. If they drenched, what was the proportion of the herd treated (<25%, 25-75%, >75%, 100%),
4. Were calves and heifers drenched with a product specifically targeting liver fluke,
5. Were they interested in attending a workshop or seminar on liver fluke, and
6. Were they interested in being part of a further study of the effect of liver fluke on milk production in their herd and to provide their contact details if they were interested.

A blank section was provided for the farmer to add any other comments concerning liver fluke.

3.2.6 Statistical analysis

Survey questionnaire data and IDEXX ELISA SP% results were entered into two separate Excel spreadsheets (Microsoft®), with herds identified by their Westland Milk Co-operative milk supplier number or left blank if the author of the survey responses preferred it withheld.

The weighted Kappa test was used to measure the agreement in BME categorisation between the first and second sampling for the 99 farms sampled twice in the autumn one week apart and between the March 2017 and October 2017 samplings for those farms sampled twice 6 months apart. The Kappa statistic varies from 0 to 1, where 0 - 0.2 indicates *slight agreement*, 0.2-0.4 indicates *fair agreement*, 0.4-0.6 indicates *moderate agreement*, 0.6-0.8 indicates *substantial agreement*, and 0.8-1.0 indicates *almost perfect agreement*.

The IDEXX ELISA results were summarised by region, for the autumn and spring samplings, and the proportion of farms in each region having an estimated production limiting level of infection of ≥20% herd prevalence. The change in IDEXX ELISA SP% from autumn to spring sampling was also summarised by region.

Maps showing the spatial distribution of West Coast dairy farms bulk milk tank tested in March and the spatial distribution of survey responses indicating herd drenching of cows were made using a terrain map downloaded from Google maps (https://www.google.co.nz/maps?hl=en).

Pearson's Chi squared was used to test whether there was a significant association between farmer drench choices and their IDEXX ELISA result in autumn and spring, for those farmers who completed the survey questionnaire and gave their supply number.

All analyses were carried out using R studio (RStudio Team (2020). RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/).

3.3 Results:

3.3.1 Repeatability of a BME

The weighted kappa analysis of 0.84 (95% CI 0.78- 0.91) showed *almost perfect agreement* between the two autumn BMT samples taken one week apart in 99 herds (Table 3.2). The mean SP% for the two testing points were 115 and 116%. However, there was one farm with an irregular result being classified as *strong* in sample one and *no or very weak* in sample two.

Table 3.2 IDEXX ELISA category of 99 herds sampled twice over a two-week period in the autumn.

		Sample 2			
		No or very weak	Low	Medium	Strong
Sample 1	No or very weak	27	6	0	0
	Low	1	7	3	0
	Medium	0	1	4	4
	Strong	1	0	4	41

3.3.2 Autumn BME Results

Sixty-nine percent (298/430) of the autumn (March) bulk milk samples (n=430), had antibodies present against *F. hepatica* with 248/430 (58%) having an SP% result in the *medium* or *strong* category suggesting ≥20% cows infected on those farms (Table 3.3).

Table 3.3 The number and percentage of herds in each IDEXX diagnostic category in the autumn.

	No or very weak	Low	Medium	Strong	Total
Number of herds in each category	132	50	62	186	430
Percentage of herds in each category	31%	12%	14%	43%	

There were 11/18 (61%) regions with at least half the herds tested that had results consistent with production limiting infections with >20% of cows affected (Table 3.4). Of the farms from the West Coast, 63% 248/395 have an SP% result in the *medium* or *strong* category suggesting ≥20% cows infected on those farms. All 35 farms sampled from Canterbury were in the *no or very weak* category. The mean bulk milk IDEXX SP% for all herds tested in the autumn was 116.

Table 3.4 Percentage of autumn sampled herds (n=430) in each IDEXX ELISA diagnostic category by region and percentage with production limiting infection (>20% cows infected) and the number of farms sampled in each region.

Region	Percentage of Herds in each IDEXX ELISA Category				Percentage of Herds with production limiting infection	Number of Herds
	No or very weak	Low	Medium	Strong		
West Coast	25	12	16	47	63	395
Karamea	7	7	13	73	86	30
Westport and Seddon	5	10	23	62	85	39
Barrytown	0	0	33	67	100	3
Inangahua and Shenandoah	56	22	6	17	23	18
Reefton	62	19	8	12	20	26
Maruia and Springs Junction	60	13	7	20	27	15
Grey Valley (Greymouth to Reefton)	46	18	24	13	37	68
Bell Hill, Haupiri, Nelson Creek	21	29	14	36	50	14
Inchbonnie and Rotomanu	23	14	18	45	63	22
Kumara and Taramakau	40	30	20	10	30	10
Arahura and Chesterfield	10	10	10	70	80	10
Kokotahi and Kowhitirangi	9	6	13	72	85	54
Ross	0	0	0	100	100	3
Waitaha and Bold Head	0	9	36	55	91	11
Harihari and Evans Creek	6	6	24	65	89	34
Whataroa and Te Aho	6	10	0	84	84	31
Fox and Franz	71	14	0	14	14	7
Canterbury	100	0	0	0	0	35

The spatial distribution of sampled herds for March 2017 (Table 3.4) is shown in Figure 3.2.

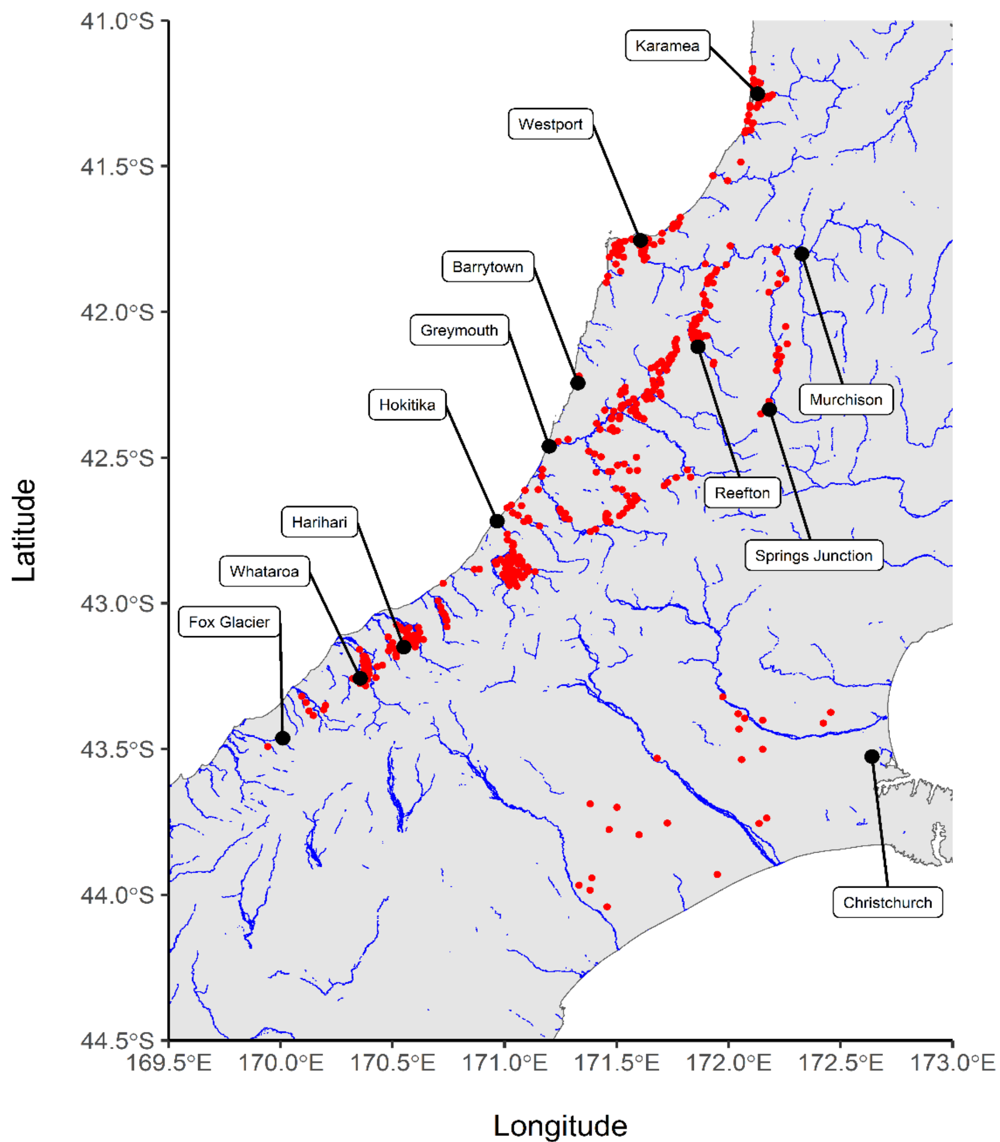

Figure 3.2 Spatial distribution of sampled farms, red dots, superimposed on a map of the major rivers of the area.

3.3.3 Spring BME Results

Sixty-four percent (256/403) of the spring (October) bulk milk samples (n=403), had antibodies present against *F. hepatica* with 185/403 (46%) having and SP% result in the *medium* or *strong* category suggesting ≥20% cows infected on those farms (Table 3.5).

Table 3.5 The number and percentage of herds in each IDEXX diagnostic category in the spring.

	Negative	Low	Medium	Strong	Total
Number of herds in each category	145	73	85	100	403
Percentage of herds in each category	36%	18%	21%	25%	

There were 10/18 (56%) regions with at least half the herds tested that had results consistent with production limiting infections (Table 3.6). Of the farms from the West Coast, 49% 183/377 have an SP% result in the *medium* or *strong* category suggesting ≥20% cows infected on those farms. Of the 26 farms sampled from Canterbury 24 were in the *no or very weak* category and 2 were in the *medium* category (Table 3.6). The mean bulk milk IDEXX SP% for all Herds sampled in the spring was 88.

Table 3.6 Percentage of spring sampled herds in each IDEXX ELISA diagnostic category by region and percentage with production limiting infection (>20% cows infected) and the number of herds sampled in each region.

Region	Percentage of Herds in each IDEXX ELISA Category				Percentage of Herds with production limiting infection	Number of Herds
	No or very weak	Low	Medium	Strong		
West Coast	33	19	22	27	49	377
Karamea	7	24	10	59	69	29
Westport and Seddon	17	14	33	36	69	36
Barrytown	33	0	67	0	67	3
Inangahua and Shenandoah	60	25	5	10	15	20
Reefton	74	13	4	9	13	23
Maruia and Springs Junction	50	21	21	7	29	14
Grey Valley (Greymouth to Reefton)	51	37	8	5	12	65
Bell Hill, Haupiri, Nelson Creek	60	7	27	7	33	15
Inchbonnie and Rotomanu	36	5	41	18	59	22
Kumara and Taramakau	60	30	10	0	10	10
Arahura and Chesterfield	0	20	30	50	80	10
Kokotahi and Kowhitirangi	17	17	32	34	66	47
Ross	0	33	0	67	67	3
Waitaha and Bold Head	18	18	9	55	64	11
Harihari and Evans Creek	12	9	32	47	79	34
Whataroa and Te Aho	4	14	43	39	82	28
Fox and Franz	71	14	0	14	14	7
Canterbury	92	0	8	0	8	26

3.3.4 Comparison of Autumn and Spring results

There were 388 herds with BTM ELISA results for both the Autumn and Spring. When comparing the results between autumn and spring, the number of herds in the *strong* category decreased 59% from 166 to 98, *medium* increased 42% from 57 to 81, *low* increased 53% from 45 to 69 and *no or very weak* increased 17% from 120 to 140 (Figure 3.3).

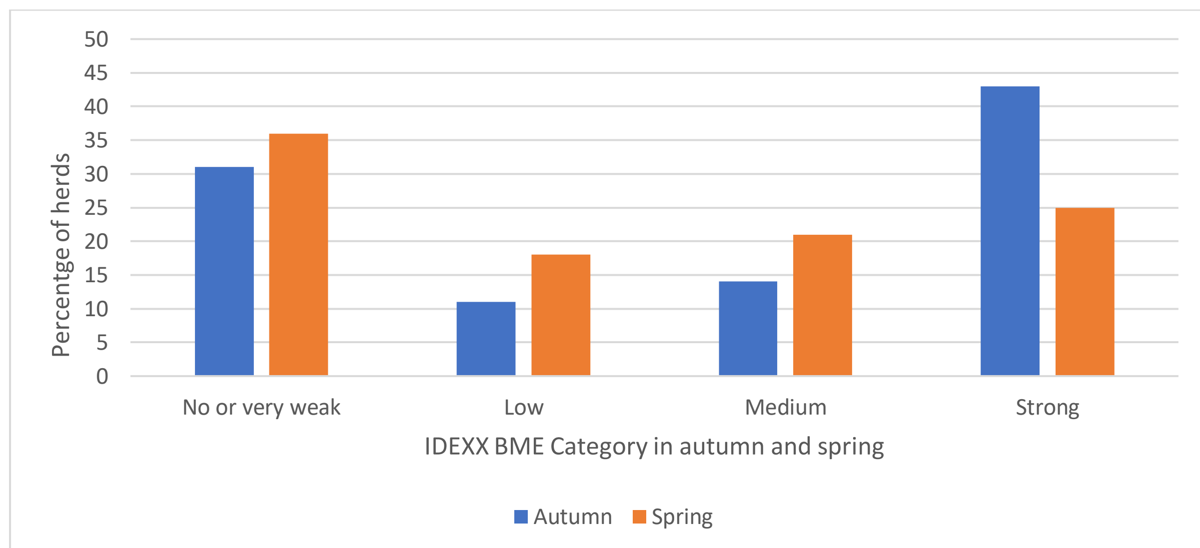

Figure 3.3 The percentage of herds in each diagnostic category in the Autumn and Spring.

The weighted kappa = 0.58 (95% CI, 0.52 to 0.64), $p < 0.0001$ showing *moderate agreement* between the autumn and spring IDEXX category diagnosis of 388 herds sampled on both occasions. Of these herds, 226/388 (58%) had the same infection category at both sampling points (Table 3.7), 34/388 (9%) had increased in category and 128/388 (33%) had decreased in category. Of the 120 *no or very weak* farms in the autumn, 23/120 (19%) tested in a positive category in the spring and of the 268 farms in a positive category in autumn 43/268 (16%) tested *no or very weak*.

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Table 3.7 IDEXX ELISA category of 388 herds sampled both in the autumn and spring.

		Spring BME Category			
		No or very weak	Low	Medium	Strong
Autumn BME Category	No or very weak	97	11	10	2
	Low	18	20	6	1
	Medium	18	17	18	4
	Strong	7	21	47	91

Almost all regions except two had a decrease in the mean IDEXX SP% from autumn to spring, the two regions that the mean IDEXX SP% increased were "Canterbury" and "Maruia and Springs Junction". However, even with the increase these regions were still classed as *no or very weak* (Table 3.8).

Table 3.8 Percentage of herds in each region with production limiting infection of F. hepatica in Autumn and Spring, the mean IDEXX SP% and the percentage decrease of SP% for each region between the two sampling periods.

	Production limiting infection Autumn	Production limiting infection Spring	Mean SP% Autumn	Mean SP% Spring	Percentage decrease of SP%
West Coast	63%	49%	125	93	26
Karamea	86%	69%	58	41	29
Westport and Seddon	85%	69%	164	113	31
Barrytown	100%	67%	180	71	61
Inangahua and Shenandoah	23%	15%	128	93	27
Reefton	20%	13%	48	36	25
Maruia and Springs Junction	27%	29%	46	50	-9
Grey Valley (Greymouth to Reefton)	37%	12%	67	43	36
Bell Hill, Haupiri, Nelson Creek	50%	33%	108	57	47
Inchbonnie and Rotomanu	63%	59%	179	141	21
Kumara and Taramakau	30%	10%	68	35	49
Arahura and Chesterfield	80%	80%	169	151	11
Kokotahi and Kowhitirangi	85%	66%	164	115	30
Ross	100%	67%	229	174	24
Waitaha and Bold Head	91%	64%	154	142	8
Harihari and Evans Creek	89%	79%	173	140	19
Whataroa and Te Aho	84%	82%	179	144	20
Fox and Franz	14%	14%	50	56	-12
Canterbury	0%	8%	4	14	-250

3.3.5 Production Limiting Infection in herds sampled in both the Autumn and Spring.

Of the 223/388 herds that had a prevalence of infection ≥20% as indicated by the IDEXX diagnostic category, indicating a production limiting infection in the autumn, 160/223 (72%) remained production limiting while 63/223 (28%) decreased to an infection category that was not considered production limiting. Of the 165/388 herds that did not have a production limiting infection, 19/165 (12%) moved up to this category in the spring. A total of 160/388 (41%) of herds had a production limiting herd prevalence of infection at both sample points.

3.3.6 Survey questionnaire

A total of 156 completed questionnaires were returned, representing 168 herds, with 161/168 (96%) of herds having a corresponding farm supply number supplied by the respondent. However, not all questions were answered by some farmers.

A total of 141/167 (84%) respondents were aware of liver fluke being present on the farm in the previous five years. 115/168 (68%) drenched cows with an anthelmintic specifically targeting liver fluke and 108/115 (94%) drenched all the cows.

Of the respondents, 161 provided a milk supplier number of which 157/161 (98%) herds were from the West Coast and 4/161 (2%) were from Canterbury. Of these herds 136/161 (84%) were aware of liver fluke being present on the farm in the previous five years. On 113/161 (70%) herds, cows were drenched with an anthelmintic specifically targeting liver fluke, of which 107/113 (95%) drenched all of the cows. Of the 77 *strong* category herds, 63/77 (82%) used a liver fluke drench and 62/63 (98%) drenched all the cows. Of the 40 *no or very weak* category herds, 13/40 (33%) used a liver fluke drench and 9/13 (69%) drenched all the cows.

Drenching cattle less than one year of age with an anthelmintic specifically targeting liver fluke occurred on 106/154 (69%) of farms and drenching cattle aged one to two years on 111/156 (71%).

3.3.7 The effect of drenching on farms that completed the questionnaire and provided their milk supplier number.

There was a strong association between drenching cows or not in the autumn and the BME category in the autumn ($\chi^2 = 31.1$, $p<0.0001$), with herds where cows were drenched tending to have a *strong* category result, whereas herds where cows were not drenched were more likely to be *no or very weak* (Table 3.9).

Table 3.9 The 150 herds where cows were drenched or not drenched in the autumn and the corresponding BME Autumn category for that herd. The percentage in brackets indicate what percentage of either drenched or not drenched herds were in each diagnostic category.

Autumn BME test category	Drench	Not drench	Total
No or very weak	13 (12%)	25 (56%)	38 (25%)
Low	17 (16%)	4 (9%)	21 (14%)
Medium	17 (16%)	3 (7%)	20 (13%)
Strong	58 (55%)	13 (29%)	71 (47%)
Total	105	45	150

There was a strong association between drenching cows or not in the autumn and the BME category in the spring (χ^2= 19.7, p=0.0001). Herds that were drenched tended to be more evenly distributed through the categories whereas herds where cows were not drenched were more likely to have a *no or very weak* category result (Table 3.10).

Table 3.10 The 150 herds where cows were drenched or not drenched in the autumn and the corresponding BME Spring category for that herd. The percentage in brackets indicate what percentage of either drenched or not drenched herds were in each diagnostic category.

Spring BME test category	Drench	Not drench	Total
No or very weak	24 (23%)	27 (60%)	51 (34%)
Low	21 (20%)	6 (13%)	27 (18%)
Medium	27 (26%)	6 (13%)	33 (22%)
Strong	33 (31%)	6 (13%)	39 (26%)
Total	105	45	150

Of the 105 herds where cows were drenched in the autumn (Table 3.11), 53/105 (50%) had the same infection category in the spring, 7/105 (7%) increased in category and 45/105 (43%) decreased in category. Of the 13 *no or very weak* herds in the autumn, 3/13 (23%) tested in a positive category in the spring and of the 92 herds in a positive category (*low, medium or strong*) in the autumn, 14/92 (15%) tested *no or very weak* in the spring. When compared, the category in the spring was quite different to that of the autumn for herds where cows were drenched (χ^2 = 12.8, p=0.005). The mean SP% of herds where cows were drenched was 142 in the autumn and 105 in the spring.

Table 3.11 Herds where cows were drenched and had a Bulk Milk ELISA in both Autumn and Spring (n = 105).

		Spring				
		No or very weak	Low	Medium	Strong	Total
Autumn	No or very weak	10	2	1	0	13
	Low	7	7	3	0	17
	Medium	6	6	4	1	17
	Strong	1	6	19	32	58
	Total	24	21	27	33	105

Of the 45 herds that did not drench in the autumn (Table 3.12), 31/45 (69%) had the same infection category at both sampling points, 2/45 (4%) increased in category and 12/45 (26%) decreased in category. Of the 25 *no or very weak* herds in the autumn, 2/25 (8%) tested in a positive category in the spring and of the 20 farms in a positive category 4/20 (9%) tested *no or very weak*. When compared, the categories in the spring were not different to that of the autumn for herds where cows were not drenched (χ^2 = 4.1, p=0.256).

Table 3.12 Herds where cows were not drenched and had a Bulk Milk ELISA in both Autumn and Spring (n=45).

		Spring				
		No or very weak	Low	Medium	Strong	Total
Autumn	No or very weak	23	2	0	0	25
	Low	3	1	0	0	4
	Medium	1	1	1	0	3
	Strong	0	2	5	6	13
	Total	27	6	6	6	45

For those herds where cows were drenched in the autumn and had a production limiting infection identified in the autumn testing (n=75, Table 11), the mean SP% decreased from 186% to 133% in the spring. However, and similarly, there was also a decrease in the mean SP% for herds with production limiting infection in the autumn where cows were not drenched (n= 16) from 194% to 144% in the spring (data not shown).

3.4 Discussion:

These results emphatically support the findings from previous studies (Charleston *et al.* 1990; Haydock 2016) that liver fluke is indeed a common parasitic infection of dairy cattle on the West Coast of South Island, New Zealand. Clustering of herds based on intensity of infection was evident in both seasons with the IDEXX BME proven to be a highly repeatable test. Farmers on the West Coast are aware of liver fluke with many using liver fluke specific anthelmintics.

3.4.1 Repeatability of the IDEXX BME test

The *almost perfect agreement* weighted kappa analysis demonstrates that the IDEXX BME is a highly repeatable test, when used in dairy herds to detect infection with liver fluke. The one herd with a spurious result (*strong* in Week 1 and *no or very weak* in Week 2) may have removed cows that had high antibody titres between the sampling periods or there may have been an error in data recording such that the herd supply number was incorrectly recorded for one of the weeks. That result aside, farmers and their advisors should feel confidence in the repeatability of this test.

The test characteristics of the IDEXX BME ELISA were not assessed in this study. However, the Canterbury herds provided a useful negative control in the autumn and spring sampling, as this region is not considered to have endemic liver fluke infection, being drier and having free draining soils that do that require humping and hollowing. In the autumn, all 35 herds in Canterbury were *no or very weak* category results and in the spring 24/26 (92%) were *no or very weak* with 2/26 (8%) having a *medium* category infection. The introduction of intermediate host snails infected with liver fluke onto the farms grazed by these two herds between autumn and spring 2017 is highly unlikely, however the introduction of cows carrying liver fluke infection is entirely possible. This could be easily accomplished by purchasing or moving cows that have spent time grazing on farms with liver fluke infection as some farmers own herds in both regions and may move cattle between them, although there are no data to support this hypothesis. The finding that the Canterbury herds were mainly negative further

validates the use of the IDEXX BME. Further testing was not undertaken to determine if this infection persisted or to monitor the antibody titre over the remaining lactation in the two Canterbury herds that returned *medium* infections in October 2017.

3.4.2 Prevalence of liver fluke infection

Approximately half of all the herds tested had a herd prevalence of infection ≥20%, which will likely be production limiting, causing financial consequences to both those dairy farmers and to Westland Milk Co-operative. These financial impacts are even further exacerbated by the finding that in herds with a high prevalence of infection, the use of anthelmintics targeting liver fluke in the autumn had little impact on the herd prevalence of infection in the following spring.

The findings that 69% and 61% of the total herds tested had a positive BME test in the autumn and spring, respectively, should be sufficient evidence for farmers, advisors, and veterinarians to appreciate the significant impact liver fluke could be having on West Coast dairy cow health throughout the year. The five-month period from possible anthelmintic treatment and the spring sample is sufficient for antibody titres to have declined (Castro *et al.* 2000; Munita 2019) indicating that cows became reinfected, assuming the treatment was effective. The importance of liver fluke to individual farmers is further evidenced by 85% of farmers reporting that they had been aware of the presence of liver fluke on their farm for the past five years.

3.4.3 Geographical distribution of liver fluke infection.

Sustaining an endemic infection of liver fluke requires a suitable habitat for the intermediate host snails. It has been observed that snail population density and intensity of infection in the definitive host are not directly related, with immature snails being more prone to infection with miracidium than adults (Boray 1963; Kendall and Ollerenshaw 1963; Harris and Charleston 1976; Boray *et al.* 1985). In regions where environmental conditions are suitable for snail egg survival and hatching then there is a prolonged supply of young snails suitable for miracidial infection. Large snails may produce ten times more redia than small snails (Rowcliffe and Ollerenshaw 1960b) so a healthy habitat for snails with moderate temperatures, adequate moisture and a food supply are all important to maintain the population.

The spatial clustering of herds seen in this study with *strong* and *no or very weak* infection category is also a feature of studies in Denmark (Olsen *et al.* 2015), the United Kingdom (McCann *et al.* 2010), Northern Ireland (Byrne *et al.* 2018), Ireland (Selemetas *et al.* 2014), Sweden (Novobilsky *et al.* 2015), Mexico (Villa-Mancera and Reynoso-Palomar 2019) and Germany (Fanke *et al.* 2017). In those studies, some related causative factors that are similar include moisture over the summer, number of days when rain occurred, temperature, flooding and presence of wetlands. Some causative factors in those studies that were considered unrelated to the present study included days spent grazing pasture, month of turnout and the percentage of pasture in the diet.

The wide range of levels of infection in this study, with 0 – 100% of herds testing positive within regions is much greater than in Germany (Kuerpick *et al.* 2013b) but is similar to north/central Portugal (Conceicao *et al.* 2004) and the United Kingdom (McCann *et al.* 2010). This supports the need to approach liver fluke infection from a regional basis using liver fluke infection history and climate data

to predict disease risk. It is noticeable that the Canterbury farms had very low levels of infection which is consistent with the environmental requirements to sustain a population of suitable snails due to the climate being too hot and dry particularly over the summer period. Farmers in Canterbury are more concerned about soil moisture deficits reducing pasture growth, in stark contrast to West Coast farmers who are more concerned about removing excess water from rainfall. The geographical clustering does suggest that some regions are a more suitable habitat for the intermediate host snails and further study is required to determine why these regions have a higher prevalence of infection and to compare these results to liver fluke infection models to assess the accuracy of predicting infection. Modelling of the climatic conditions that favour snails and comparing this to the herd liver fluke infection intensity was not in the scope of this study but would be a valuable use of the current dataset.

3.4.4 Liver fluke infection at a level likely to be production limiting

In this study, we used a cut point of ≥20% of cows infected herd prevalence to define a production limiting infection. This is slightly lower than that used by other studies where a production limiting effect was considered to occur when 25% or more of the herd was infected (Vercruysse and Claerebout 2001; Charlier *et al.* 2007). This slight disparity was due to the interpretation of the IDEXX BME SP% which gives four infection categories with the *medium* and *strong* categories equating to a herd prevalence ≥20% infected cows. We believe that 20% and 25% are sufficiently similar that prevalence results presented in this study and those presented in international studies can legitimately be compared. In the current study 57% of herds in the autumn and 46% in the spring had infection at a level likely to be production limiting. For the subset of herds from the West Coast, 63% in the autumn and 49% in the spring had infection at a level likely to be production limiting being: lower than the 72-86% reported in England (McCann, 2010) and Wales (Salimi-Bejestani *et al.* 2005a; McCann *et al.* 2010); but similar to other studies in Ireland, northern Germany, England, Belgium and Northland in New Zealand (Salimi-Bejestani *et al.* 2005a; McKay 2008; Bennema *et al.* 2009; Kuerpick *et al.* 2012b; Kuerpick *et al.* 2012a; Byrne *et al.* 2018); and greater than Switzerland, Austria and Sweden (Novobilsky *et al.* 2015; Kostenberger *et al.* 2017; Frey *et al.* 2018). Notwithstanding any differences in ELISA tests and test interpretation used in the aforementioned studies, it is likely that the prevalence of liver fluke infection on the West Coast is high by international standards.

Of the herds with a production limiting infection identified in the autumn sampling, 72% had infection at this level again in the spring despite any management interventions to remove the parasite or reduce reinfection. This is similar to the findings in Germany (Kuerpick *et al.* 2012a) where liver fluke control measures such as pasture management and anthelmintic use were considered to be ineffective. Unfortunately, the dry period is the only opportunity West Coast dairy farmers have to treat dairy cows for liver fluke, but if drenching in the late autumn is not effective at interrupting the liver fluke lifecycle then this practice needs to be reflected upon.

There was no noticeable difference in the mean SP% decrease from autumn to spring for farms that had a production limiting infection identified in the autumn irrespective of whether the cows were drenched or not. If the drench was effective and cows did not become reinfected it would be expected that the BME SP% would have decreased by the spring (Munita 2019). This further indicates that a treatment for fascioliasis in the autumn was relatively ineffective in controlling liver fluke. This result is contrary to that found by Kostenberger *et al.* (2017) where herds with high optical density ratio

(ODR) had a significant decrease between seasons when drenched whereas herds with low ODR did not. In the Kostenberger *et al.* (2017) study, although the cows were drenched at dry off, as for the West Coast cows, they were not seasonal calving herds and the cows themselves were housed for substantial periods of the year. The Kostenberger *et al.* (2017) study clearly showed that drenching can be effective when given at dry off, but only if the cows are also taken off pasture. With no off-pasture capacity for West Coast cows then efforts should be possibly directed at making the pasture safer or using other feeding methods like feed pads.

3.4.5 Farmer drenching practices and herd reinfection

The use of anthelmintics is a common feature of liver fluke management plans in many countries (Knubben-Schweizer *et al.* 2010; Charlier *et al.* 2012; Bloemhoff *et al.* 2014; Selemetas *et al.* 2015) and this practice also appears to be common on the West Coast of New Zealand with 68% of farmers who returned the questionnaire indicating they drench adult cows for liver fluke. Anthelmintics in New Zealand with registered efficacy claims against liver fluke in cows producing milk for human consumption, have legal milk withholding periods ranging from 14 to 91 days, which means that the only suitable times to drench adult cows for liver fluke is at the end of lactation or during the non-lactating dry period. All anthelmintics targeting liver fluke also contain actives that have some activity against other gastrointestinal parasites.

In this study, the *strong association* between whether the farmer drenched to treat for fascioliasis or not in the autumn, and the autumn BME category (Table 3.9) indicates that farmers have an awareness of liver fluke infection in their herd and they perceive drenching as an important part of a liver fluke management plan. In that year, the results letter and survey questionnaire were sent at the same time and this may have further influenced the decision to drench. Of the farmers whose herds had a liver fluke positive result, most were likely to drench cows, with almost all drenching the entire herd. This suggests that farmers are seeking the reassurance of treating all cows possibly due to the inability to visually assess which cows are infected. The concern about the potential impacts of liver fluke as well as the commercial marketing of anthelmintics to dairy farmers during the dry-off period may also be the reason why 13/38 (34%) herds with negative tests still decided to treat, a not insignificant cost, although the question may have been interpreted to include any drenching over the previous five years.

Interestingly, 18% of the surveyed farmers with a *strong* category autumn result did not drench their cows (Table 9). This could be due to perceived lack of an economically significant result, management implications of the milk withholding periods, lack of facilities to administer anthelmintics, general lack of awareness of liver fluke or other factors. At the spring testing (Table 10), of these 13 herds; 6 were still *strong*, 5 *medium* and 2 *low,* so for 54% of the herds the category of infection had decreased without any anthelmintic use. This is very similar to the 45% of the *strong* herds that drenched that also had the category of infection decrease by the spring (Table 10). This result indicates that for herds with a high prevalence of infection, drenching cows in the autumn does not appear to have an impact on the fluke population, which may be due to efficacy of the anthelmintic against immature flukes, anthelmintic resistance or reinfection after treatment. While anthelmintic resistance to triclabendazole has been reported in New Zealand and around the world (Fairweather and Boray 1999; Hassel and Chapman 2012; Elliott *et al.* 2015; Hanna *et al.* 2015; Novobilsky and Hoglund 2015; Kelley *et al.* 2020; Alvarez *et al.* 2022) this was not investigated in this study.

Our limited understanding of the liver fluke lifecycle in New Zealand is based on studies conducted in the Manawatu, a southern region of the North Island, that found metacercariae build-up in the autumn but they do not survive the winter (Harris and Charleston 1976). Unfortunately, no similar studies have been conducted on the West Coast. The evidence from this study would indicate that a parallel drop in infection prevalence occurred on farms that drenched in the autumn and on farms that didn't. This clearly is a paradox but likely indicates that on some farms, cows are becoming infected again by October (spring) possibly due to overwintering of embryonated eggs, overwintering of infected snails, metacercariae being infective for some or all of the winter period, reinfection of cows soon after treatment in the early winter or immature flukes continuing development due to being unaffected by the anthelmintic choice.

It takes an average of 114 days for redia larval development in *P. columella* at 24°C (Boray 1978), and the annual mean monthly temperatures on the West Coast are lower at 5-18°C so development here would take longer than this average. Thus, overwintered eggs are more likely to result in summer or autumn infection of cattle, rather than spring infection. Embryonated eggs that overwintered and released miracidia to infect snails did not result in earlier cercarial production compared to snails infected by miracidium developed from eggs in the spring (Luzon-Pena *et al.* 1994). Similarly, eggs produced by liver flukes that had survived either due to anthelmintic resistance or being outside of the efficacy spectrum of the drug at the time of treatment, would be more likely to result in summer or autumn infection of cows. This current study did not attempt to determine what anthelmintics were used by farmers but the injectable options are only effective against adult flukes and it is likely that cows which were infected by immature flukes at the time of treatment will have continued their maturation.

In studies where metacercariae have been shown to overwinter, their viability rapidly declined through the spring period so their impact was thought to be low (Ollerenshaw 1971; Luzon-Pena *et al.* 1994). Infected snails can survive a mild winter (Luzon-Pena *et al.* 1994) and redia development can recommence once the snail is active in the spring but this is also more likely to result in summer infection of cows. In addition to fluke juvenile stages being outside of the spectrum of efficacy of any anthelmintic, continuing their development, it is quite possible that cows become reinfected soon after treatment as the anthelmintics have no residual action. Autumn on the West Coast is generally wet (farmer pers comms) and this would support autumn and early winter infection of cows. The enforced timing of drenching of cows at the end of lactation, through legal withholding periods on anthelmintics, does not appear to be an effective measure to disrupt the parasite lifecycle on farm. More regular monitoring of herds over the lactation and the dry period would indicate when infection occurs to identify timing of parasite infection. Further investigation into the impact of these possible causes is warranted to help determine the reason for the apparent lack of impact that drenching had on herds with liver fluke infection detected in the autumn.

Any change in liver fluke infection in herds in this study from autumn to the following spring could be related to: removal of infected cull cows at or near the end of the lactation period; the addition of heifers or "carry-over" adult cows at the start of lactation; anthelmintic treatment in the "dry" (non-lactating) period; seasonal factors affecting the intermediate host snails and lifecycle stages of liver fluke in the environment; and the cows being grazed on forages with either a lower or greater metacercarial infectious load compared to the lactation period.

The IDEXX BME test detects antibodies 2 weeks post infection (wpi) with the antibody concentration in milk mirroring that of serum but at a much lower concentration of 5% (Farrell *et al.* 2004; Mezo *et al.* 2010a). Antibody concentration declines after successful treatment but may still be present 90 days later (Munita, 2019), therefore any antibodies detected in the spring are unlikely to be a residue from a successfully treated autumn infection.

Liver fluke infection in the West Coast was common in this study and the similarity of infection in both seasons (Table 3.7) is similar to one study in Ireland (Byrne *et al.* 2018) although different to another in that country (Bloemhoff 2015) where the rate of infection increased more notably in the autumn. While the findings in the current study suggests a consistent infection rate is occurring regardless of season, in studies where liver fluke prevalence is lower (Conceicao *et al.* 2004) seasonal variation can be more considerable, relating to conditions that suit proliferation of the intermediate host snails. All of the cows in this study were spring calving, pasture fed, dried-off in the autumn, with a non-lactating period in the winter, and never housed (off pasture); features which may differ considerably to overseas studies. An increase in disease prevalence between seasons has been associated with grazing management and increased days at pastures in several studies (Novobilsky *et al.* 2015; Selemetas *et al.* 2015; Arenal *et al.* 2018; Takeuchi-Storm *et al.* 2018). As pasture is the dominant if not exclusive source of forage for farms in the present study, it appears that metacercariae infection may be occurring through the winter suggesting liver fluke infection is a year-round continuous challenge for farms in some regions of the West Coast. It should be noted that while it appears that the conditions in 2017 supported liver fluke survival, this may not be the case every year due to variable climatic events, such as La Niña.

The herds that tested negative in the autumn were likely to remain negative, but we did not investigate why. This may be due to natural features of the farm or interventions made by the farmer to disrupt snail habitat or access to metacercariae. While positive herds overall saw a decrease in SP%, this did not necessarily result in a change of disease category. This result is similar to the findings on north German farms that were monitored for liver fluke over two years (Kuerpick *et al.* 2012a). In a study of Danish herds, the authors determined that the use of the IDEXX category was more useful than simply comparing SP% values (Takeuchi-Storm *et al.* 2021).

3.4.6 Summary

Taken together, the results of this current study have determined that liver fluke infection is common in dairy herds on the West Coast with almost 50% having an infection at a level that likely impacts production. The results also suggest that current intervention practices are having a limited impact on the *F. hepatica* life cycle in dairy cows in the West Coast region of New Zealand. Further studies are needed to gain a better understanding of the epidemiology of the life cycle of liver fluke in this region so that control measures and disease modelling can be better used to control the disease. Furthermore, the presence of liver fluke infection that will likely impact production on approximately half of the dairy herds in the study validates the need to determine the economic cost of this infection in terms of milk production parameters. Further studies in this thesis will investigate the impacts of liver fluke infection on milk production parameters at the individual cow level in both a cross-sectional and a longitudinal study. Determining the economic cost of liver fluke will allow farmers to decide whether to investigate ways to reduce the intensity of infection in their herd or accept the impact.

3.5 Ethics permission

This study was performed under the approval of Massey University Animal Ethics Committee, Protocol MUAEC15/92.

3.6 Supplementary Materials

Supplementary materials are contained in Section 3.2.2 and 3.2.3.

Chapter 4. Cross Sectional study of associations between liver fluke infection on milk production on eleven farms.

4.1 Introduction

Liver fluke infection has been shown to cause significant production losses in farmed ruminant as a direct result of hepatobiliary pathology caused by migration and residence of the flukes (Charlier *et al.* 2007) with lesser impacts due to secondary factors including blood loss (Dawes 1964), hypoalbuminaemia (Anderson *et al.* 1977) and enhancing the impacts of secondary infections (Aitken *et al.* 1978; Claridge *et al.* 2012).

Milk production (litres or kilograms) losses due to the presence of liver fluke infection or improved production after treatment has been reported as 3-15% in Europe (Black and Froyd 1972; Charlier *et al.* 2007; Mezo *et al.* 2011; Charlier *et al.* 2012; Howell *et al.* 2015; Kostenberger *et al.* 2017; May *et al.* 2020; Novobilsky *et al.* 2020; Springer *et al.* 2021; Takeuchi-Storm *et al.* 2021) and 16-32% in the central Americas (Arenal *et al.* 2018; Villa-Mancera and Reynoso-Palomar 2019). Impacts on the milk constituents (milk fat, lactose or protein) of 0.06 kg/cow/day or 0.06-2.2% (Charlier *et al.* 2007; Charlier *et al.* 2012; Kostenberger *et al.* 2017; May *et al.* 2020) have also been reported. To develop a model to predict the impact of liver fluke infection on cows in Switzerland, Schweizer *et al.* (2005) determined that infected herds had a 10% reduction in milk yield (litres/cow/day). Not all studies however have been able to determine a statistically significant impact on milk production (Hayward *et al.* 2021) with impacts at the cow level not always being evident at the herd level. These effects may be lost due to too few cows in the herd having a production limiting infection, even though the prevalence is high. This focuses the need for diagnostics capable of quantifying the current liver fluke infection in the herd by either sampling a subset of the herd or using pooled samples.

Studies investigating the impact of liver fluke infection on milk production either measure the impact of active infection against no infection, or flukicide treatment against no treatment in infected animals, at the individual and herd level (Chapter 1). Studies are often conducted in herds which calve all year round, which means that individual cows enter and leave the herd throughout the year and are thus at different stages of lactation when trials are conducted. For example, the milk volume, fat and protein composition of milk changes markedly over the lactation period (Silvestre *et al.* 2009) which makes measuring the effect of infection or treatment difficult to measure. Since cows will be at different stages of lactation at the time of testing, impacts on these parameters due to liver fluke infection may be apparent at the individual cow level but be masked at the herd level. This situation is very different in New Zealand, where all cows are at a similar stage in lactation due to seasonal spring calving with a greater likelihood that changes at the cow level will be apparent at the herd level also.

Another significant difference in farming systems in New Zealand is absence of housing animals over a winter period. Due to this difference, cattle can be exposed to metacercariae on pasture for most, if not all of the year, whereas housing animals removes any further potential of infection and if an anthelmintic treatment is administered, the liver has a period of recovery.

A prevalence study of liver fluke infection in herds supplying milk to Westland Milk Co-operative (Chapter 3) in the autumn and spring of concurrent lactations, revealed 69% and 64% of herds

respectively having liver fluke infection. Of the total herds sampled, 58% in the autumn and 46% in the spring had infection at a level considered to cause milk production losses (Vercruysse and Claerebout 2001; Charlier *et al.* 2008) indicating that liver fluke is likely to have a significant negative financial impact on these farms and the West Coast.

To the authors knowledge there have been no previous studies to quantify the impact of liver fluke infection in New Zealand dairy herds. International studies may not be relevant in New Zealand due to significant differences in farm practices; seasonal calving over a two-month period, cessation of lactation in the late autumn/early winter, a pasture-based diet grazed in situ, with no housing for long periods.

4.1.2 Aims of this study

The aims of this study include assessing the within herd prevalence and range of intensity of infection in dairy herds on the West Coast and assessing the accuracy of the bulk milk ELISA to the mean SP% of serum samples from cows sampled on the same day. Also, to investigate associations between liver fluke infection determined by antibody ELISAs, coproantigen ELISA and FEC assessed near the end of lactation and milk production parameters from that lactation. A sample of heifers were also sampled on some farms to determine the prevalence of liver fluke infection in this age group in comparison to the adult cows.

4.2 Materials and Methods

4.2.1 Study design

A cross-sectional study was conducted where up to 160 cows from each of 11 herds were serum sampled near the end of the lactation. All herds enrolled in the study had to conduct herd tests by LIC and make the data available. Cows present in the milking mob on the day of sampling were age identified and those in their first and second lactation were preferentially sampled with the remainder being older cows. Serum samples were analysed for the presence of anti-fasciola antibodies and milk production data was collated from the herd testing results provided by the farmer.

4.2.2 Herd selection

Farmers who had indicated in the questionnaire (Chapter 3) their willingness to involve their herd in further studies investigating the effects that liver flukes have on milk production were contacted by email and then telephone. Herds were considered suitable if they had at least 125 lactating cows and 20 two-year old heifers that would be available to blood sample in Autumn 2018, were performance monitored (herd tested) at least three times per lactation, were representative of the different geography of the West Coast including river valleys, river flats, flood prone plains and summer dry flats.

In all, nine herds (A,B,C,D,E,F,G,H,I) were enrolled and sampled in the autumn of 2018 and two herds (J,K) were resampled in the autumn of 2021. A different letter identifier for each year was used for these two herds (D=J, H=K). For coproantigen and faecal egg count (FEC) analysis, Herd A was sampled in the autumn of 2019 while herds J and K were sampled in the autumn of 2021. A unique identifier was created for each cow due to the same tag identifier being used across multiple herds by joining

the herd identifier and cow identifier using the concatenate function in Microsoft Excel (Microsoft Corporation, 2018. Microsoft Excel, https://office.microsoft.com/excel).

4.2.3 Sample Collection

From each cow up to 8mL of blood was collected from the tail vein into a serum vacutainer with a new 18g needle for each animal. Samples were collected in the autumn of 2018 or 2021 near the end of that season's lactation, using the procedure described in the standard operating procedure (Section 3.1.7) from all farms. For Herds A, J, K faecal samples were also collected by inserting a gloved hand into the rectum and faeces placed in a 70mL plastic pottle in the autumn of 2019 or 2021. Where possible, cows in their first and second lactation were preferentially sampled to increase the probability of including cows with less severe liver pathology that may be the result of repeated years of liver fluke infection.

These younger cows were identified by either the colour of the tag or the number range as per the farmers personal recording system. The day's sampling continued until up to 160 individual cow samples (A,B,D,E,G,H,I,J,K), or the entire herd was sampled (C,F). Animal identification was recorded using the ear tag number, either on a dictaphone to be transferred later to paper, or directly on paper depending on the presence of an assistant at the time of sampling.

A sample of milk from the vat was collected from the sight glass into a 70mL plastic container at the conclusion of milking on the day of blood sampling.

On days where the air temperature was low (~<10°C), the vacutainers containing blood were placed in an insulated bag for transport to the veterinary clinic to allow clotting and serum separation to occur. Otherwise, the vacutainers were allowed to stand at ambient temperature until sampling concluded and then stored in a chilled insulated carrier bag and transported to the local veterinary clinic. All vacutainers containing blood were stored at 4°C at the veterinary clinic until transported to the laboratory on that or the following day. Milk samples were placed in a chilled container for transport to the local veterinary clinic to be stored in a freezer on the same morning as collection. Faecal samples were stored at ambient air temperature until the end of sampling and then placed in a chilled container for transport to the local veterinary clinic where they were stored at 4°C until transported to the laboratory. Chilled blood and faecal samples and frozen milk samples were couriered to Massey University where the blood samples were centrifuged at 1,100 g for 15 minutes (Thermo Scientific, Heraeus Megafuge 40) and pipetted into labelled 1.5mL Eppendorf tubes. Paired samples of serum from each animal and milk were stored separately at -20°C. Faecal samples were split with one portion frozen at -20°C and the other portion chilled at 4°C until analysed for FEC.

Individual cow production data was available through LIC herd testing. Most New Zealand dairy farms that use the LIC herd testing scheme, have 4 herd tests a year in which 24 hour test-day milk data is recorded for each lactating cow in the herd. Herd testing is carried out at the morning and afternoon milking for herds that are milking twice a day, or once for herds milked once a day. It is undertaken by certified personnel from a milk testing company. The milk samples are processed at the company's laboratory and the data is entered into the farmers herd recording system (e.g. MINDA | LIC) or sent to the farmer electronically in a csv or pdf format. Data provided from the herd test includes milk volume at each sampling and total (litres), test milk lactose, protein and lactose (% and kg), milk solids and test somatic cell counts (000). This data is also used to estimate the seasonal performance of

each cow. The herd testing data was used to calculate energy corrected milk (ECM), a summary measure of the energy required to produce one litre of milk considering the milk yield and the components of fat and protein.

4.2.4 Cow Breed Categorisation

Cow breeds were classified in Table 4.2 according to the farmers records. A cross-bred cow may have a combination of Jersey, Friesian, Ayrshire or other genetics. No attempt was made to determine how each farmer classified the breed of the cows in their herd.

Table 4.1 Breed description and classification.

Breed description	Breed classification
Cross-bred	X
Jersey cross (predominantly Jersey but not purebred)	JX
Jersey	X
Friesian cross (predominantly Friesian but not purebred)	FX
Friesian	F

4.2.5 Herd testing data

Farmers were requested to provide access to their herd performance recording data for the current lactation for this study. Data was categorised by farm identifier, cow identification number, number of the herd test for that lactation (one to five) and date of the herd test, breed (Table 4), cow age (years), lactation number (1,2, 3+), IDEXX serum SP% and category and the milk production data from the herd test results.

Energy corrected milk (ECM) was calculated using the following formula (Santschi *et al.,* 2011):

$$ECM\ (kg/d) = 12.55 \times fat\ (kg/d) + 7.39 \times protein\ (kg/d) + 0.2595 \times milk\ yield\ (kg/d)$$

New Zealand herd test data is recorded as volume and not as weight. The density of milk is estimated at 1.03kg/L and although using volume instead of weight introduces a small bias, the calculation of ECM was not altered to be consistent with Mason *et al.* (2012).

4.2.6 Faecal analysis

4.2.6.1 Faecal Egg Count

The faecal egg count (FEC) technique is described in full in Section 3.1.1. Briefly, 5g of faeces are passed through the Flukefinder with water, the recovered eggs are sedimented three times and then counted at 4X magnification in a petri dish including one drop of methylene blue. The total number of eggs counted were divided by 5 to calculate eggs per gram (epg).

4.2.6.2 Coproantigen ELISA

Faecal samples were collected from the rectum and stored at -20°C for 70 (Herd J, K) to 121 days (Herd A) before analysis.

The *F hepatica* ESA concentration in the faeces were analysed using a commercial ELISA test (Bio K 201 – Monoscreen AgELISA *Fasciola hepatica*, Bio-X Diagnostics S.A. Rochefort, Belgium). The protocol is described in full in Section 3.1.6 with the following giving a brief summary. The samples and positive control (C+) (control sample provided with the kit) are duplicated in the ELISA plate provided. The optical density (OD) is read using a 450nm filter.

The net optical density of each sample and C+ are calculated by the following formula

Net OD = OD of the Ag coated well – OD of the uncoated well

The Value (Val) for each sample is calculated using the following formula

Val = (net OD sample * 100) / net OD C+

Modifications made to the manufacturer's protocol included overnight incubation of faecal samples in the dilution buffer and use of an optimised cut-off value of 1.4 based on previous studies (Brockwell *et al.* 2013; Brockwell *et al.* 2014; Novobilsky and Hoglund 2015). Coproantigen values (Val) are expressed as a percentage positive (PP) of the C+.

4.2.7 Antibody ELISA

4.2.7.1 IDEXX serum ELISA

The *F. hepatica* F2 antibody concentrations in serum and bulk milk samples were analysed using a commercial ELISA test (IDEXX Fasciolosis Verification, IDEXX Europe BV, Hoofdorp, The Netherlands) as per the manufacturer's instructions, described in full in Section 3.1.9. The colour reaction is read as an optical density (OD) at 450nm and the net extinction (NE) for each sample is calculated by subtracting the OD value that develops in the wells containing –Ag from the OD developing in wells containing +Ag to which the same sample (controls or sample) was added. The sample-to-positive percentage (SP%) is calculated by comparing the NE of the sample with the mean NE of the positive control. The SP% for each sample was calculated using the formula: SP% = 100 × (NE / NE × PC).

Test kit interpretation categorises the individual cow serum sample SP% as; *negative* (SP% ≤30), *mild positive* (30 < SP% ≤ 80), *positive* (80 < SP% < 150) and *strong positive* (SP% ≥150). For bulk milk, the ELISA test interpretation categorises the prevalence of herd infection level as; *no or very weak* (SP% ≤30), *low* with <20% cows infected (30 < SP% ≤ 80), *medium* with ≥20% cows infected (80 < SP% < 150) and *strong* with more than 50% cows infected (SP% ≥150) (Table 4.2).

Table 4.2: IDEXX SP% and test diagnostic categories for serum and milk samples.

IDEXX SP%	SP% ≤30	30 < SP% ≤ 80	80 < SP% < 150	SP% ≥150
Individual serum ELISA interpretation	negative	mild positive	positive	strong positive
Bulk milk ELISA interpretation	no or very weak	low	medium	Strong
Predicted sample prevalence		<20%	≥20% x ≤50%	>50%

For analysis the serum IDEXX SP% quartiles were calculated in R for each dataset, and the prevalence categories were numbered 1 (*negative*) through 4 (*strong positive*).

4.2.7.2 IDEXX bulk milk ELISA

The bulk milk ELISA (BME) antibody titre was determined by analysing the *F. hepatica* specific f2 antibody concentration in the bulk tank milk (BTM) milk samples using a commercial ELISA test (IDEXX Fasciolosis Verification, IDEXX Europe BV, Hoofdorp, The Netherlands) following the manufacturer's instructions which are describe in full Section 3.1.9. The colour reaction is read as an optical density (OD) at 450nm and the net extinction (NE) for each sample is calculated by subtracting the OD value that develops in the wells containing –Ag from the OD developing in wells containing +Ag. The kit contains a positive control (PC) and negative control (NC). The sample-to-positive percentage (SP%) is calculated with the formula:

$$SP\%_{sample} = 100 \times (NE_{sample} / NE_{PC}).$$

Test interpretation categorises and describes the prevalence of herd infection level based on the SP% (Table 4.2).

4.2.7.3 In-House serum ELISA

The In-House ELISA is a serum antibody test based on the method developed by Salimi-Bejestani (Salimi-Bejestani *et al.* 2005b) with the process fully described in the Section 3.1.5. In brief, liver flukes collected from naturally infected sheep were used to obtain ESA. This ESA was filtered, and the concentration determined, then used to coat the high binding ELISA plates overnight before running assays the following day to assess the reactivity of the serum samples against a positive and negative control sample. The positive control sample came from a naturally infected mixed age cow which was analysed at a commercial laboratory (NZVP Laboratory, Palmerston North) using the IDEXX Fasciolosis Verification test to have an IDEXX SP% of 244 being *strong positive* category of infection. The negative control sample is from a farm without any history of liver fluke infection in Culverden, North Canterbury, an IDEXX SP% of 2 and 4, being *negative* category of infection. The mean optical density (OD) for the positive control (C+), negative control (C-) and each sample is calculated as the mean of the two OD readings for each respectively. The modified OD for the positive control is calculated using the formula

Modified OD C+ = mean C+ OD minus mean C- OD.

The modified OD of each sample is calculated using the formula

Modified OD sample = mean sample OD minus mean C- OD.

The optical density ratio (ODR) is then calculated using the formula

ODR = (modified sample OD)/ (modified C+ OD) and expressed as a ratio.

4.2.8 Statistical Evaluation

The data structure itself was hierarchical and had 3 levels of information and potential variability, the 1st level was the repeated herd tests on individual cows, the 2nd level was the individual cows and the 3rd level was the herds from which the individual cows were randomly selected.

Models of each milk production parameter (ECM, milk fat %, milk protein %, milk lactose %) were first created for all cows from all herds using a repeated measures random effects model in R using packages 'lme4' (Bates *et al.* 2014) and 'emmeans' (https://CRAN.R-project.org/package=emmeans). The fixed effects tested in the models and retained at $p<0.05$ using the log likelihood ratio test (LRT) included DIM, lactation, breed and herd test.

DIM was calculated as the time from calving to each herd test date. DIM were centred and two polynomials were created, DIM^2 and DIM^3. Lactation was used as a proxy for cow age with cows categorised by lactation as either first, second or third and more. Cow breed was categorised by the farmers description of each cow and herd test were categories as 1,2,3 or 4. Cow nested in herd was entered as a random effect and a random slope for DIM was tested and retained at $P<0.05$ and LRT.

These milk production models were then tested to investigate the effect of fluke infection/antibody level. For each outcome variable (=all different components of milk) the measures of liver fluke infection (IDEXX SP%, In-House ODR, FEC and coproantigen value) were tested and included the test value, a binomial created of positive/negative results and quartiles of the test values. Whereas as all farms were tested for IDEXX ELISA, a reduced number were tested for In-House ELISA, FEC and coproantigen.

The random effects of herd and cow were used with model goodness of fit determined by graphing the residuals and measuring the $R^{2.}$ Value.

To determine the variability of serum IDEXX SP% within and between herds, intraclass correlation (ICC) was calculated in R.

Cohen's kappa coefficient (kappa) was used to measure inter-rater relatability of the IDEXX diagnostic categories of serum and bulk milk ELISA results from herds sampled on the same day. The mean serum IDEXX SP% of cows from the herd sampled on the day was used to assign the serum diagnostic category, and the milk sample collected on the same day was used for the IDEXX bulk milk diagnostic category as described in Table 4.2. Kappa was calculated in R, with interpretation of the values based on Landis and Koch (1977); < 0 as indicating *no agreement*, 0–0.20 as *slight*, 0.21–0.40 as fair, 0.41–0.60 as *moderate*, 0.61–0.80 as *substantial*, and 0.81–1 as *almost perfect agreement*.

4.3 Results

4.3.1 Herd code and farm geography

The herds enrolled represented a spectrum of the geography of farms representative of the West Coast. (Table 4.3)

Table 4.3 Herd code and farm geography

Herd Code	Farm Geography
A	Lakeside prone to flooding, river passes through farm
B	Well drained river flat, bordered by river on one side
C	Coastal, prone to sea surges and flooding
D	Poorly drained river flats, bordered by river, long and narrow farm
E	Well drained river flat, tendency to be dry in summer
F	Valley floor prone to flooding
G	Poorly drained valley floor
H	Poorly drained river flats, bordered by river, flood prone
I	Valley floor
J	Poorly drained river flats, bordered by river, long and narrow farm
K	Poorly drained river flats, bordered by river, flood prone

4.3.2 Cow breed

This dataset of 1538 cows was dominated by Jersey and Jersey cross cows (58%), then Friesian and Friesian cross (35%) with the remainder being Crossbred (8%) (Table 4.4).

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Table 4.4 Breed composition of the complete dataset of cows with herd testing and liver fluke data.

Breed description	Percentage of each breed
Cross bred (X)	8%
Friesian (F)	10%
Friesian cross (FX)	25%
Jersey (J)	38%
Jersey cross (JX)	20%

(percentages do not total 100 due to rounding)

4.3.3 Herd testing and serum sampling dates

The herd testing data was reported from 10/11 herds although not all cows in each herd were present at each herd test with some tested cows unable to be identified resulting in a range of 1168-1314 cow present at each test. Of these cows, 23% were in their first lactation, 25% second and 52% third or more lactations. Herd F conducted five herd tests including one in January which did not align with the timing of a concurrent test in the other herds so this January test was removed from the analysis. Herd J performed three tests over the lactation, so is absent from Test 2 (Table 4.5).

Table 4.5 Herd testing dates and serum sampling dates for each herd.

Herd	Herd test dates				Serum sampling date
	1	2	3	4	
A	27.9.2017	5.12.2017	5.2.2018	8.4.2018	11.4.2018
B	10.10.2017	4.12.2017	13.2.2018	29.4.2018	12.4.2018
C	1.10.2017	4.12.2017	13.2.2018	24.4.2017	7.5.2018
D	19.9.2017	21.11.2017	25.2.2018	9.5.2018	24.4.2018
E	2.10.2017	6.12.2017	15.2.2017	10.4.2018	9.5.2018
F	12.9.2017	9.11.2017	26.2.2018	19.4.2018	10.4.2018
G	4.10.2017	3.12.2017	29.1.2018	3.4.2018	26.4.2018
I	17.9.2017	20.11.2017	22.1.2018	27.3.2018	8.5.2018
J	13.10.2020		26.1.2021	25.3.2021	19.4.2021
K	7.10.2020	1.12.2020	3.2.2021	12.4.2021	21.4.2021

4.3.4 Milk production data and lactation models

For all herds, the mean days in milk (DIM) at each herd test were 35, 96, 169 and 228 days with a mean duration between tests of 61, 73 and 59 days (Figure 4.1).

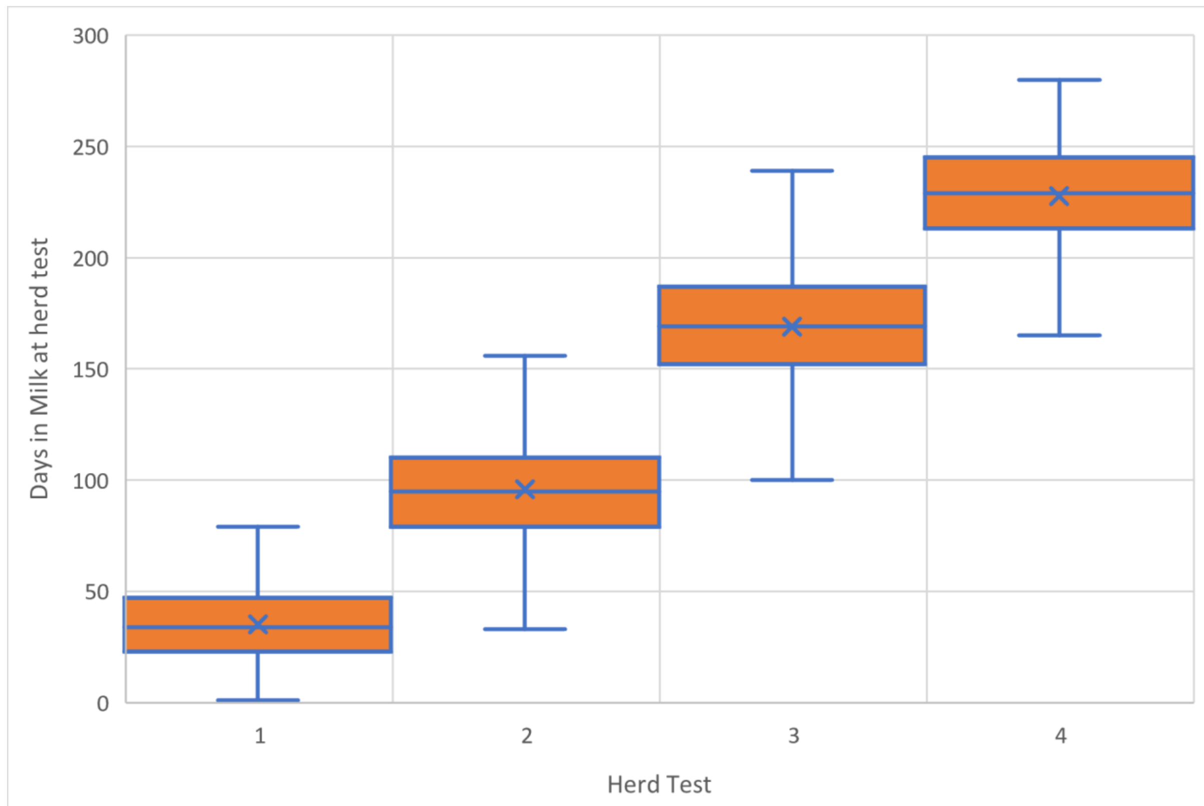

Figure 4.1 Box plots of days in milk (and mean) of all cows at each herd test.

The mean and standard errors of milk production parameters from all herd tests for all herds are presented in Table 4.6.

Table 4.6 The mean (and standard error) of milk production parameters of all herd test data from each herd.

Herd	**Mean (standard error) milk production parameters measured at each herd test**				
	Lactose %	Fat %	Protein %	MS	ECM
A	5.01 (+/-0.27)	5.18 (+/-1.04)	4.14 (+/-0.55)	1.45 (+/-0.38	19.12 (+/-5.23)
B	4.57 (+/-0.37)	5.38 (+/-1.71)	3.87 (+/-0.92)	1.32 (+/-0.43)	17.87 (+/-6.02).
C	4.77 (+/-.034)	6.64 (+/-1.56)	4.66 (+/-0.74)	1.19 (+/-0.32)	15.31 (+/-4.3)
D	4.92 (+/-0.29)	5.45 (+/-1.29)	4.32 (+/-0.64)	1.46 (+/-0.43)	19.25 (+/-5.87)
E	4.99 (+/-0.16)	5.07 (+/-0.92)	4.07 (+/-0.55)	1.48 (+/-0.39)	19.68 (+/-5.48)
F	4.94 (+/-0.21)	6.29 (+/-1.01)	4.67 (+/-1.01)	1.42 (+/-0.43)	18.32 (+/-5.68)
H	5.04 (+/-0.23)	5.43 (+/-0.92)	4.17 (+/-0.51)	1.47 (+/-0.4)	19.39 (+/-5.37)
I	4.96 (+/-0.20)	5.21 (+/-0.88)	3.98 (+/-0.44)	1.72 (+/-0.45)	22.89 (+/-6.16)
J	5.02 (+/-0.22)	5.37 +/-0.86)	4.29 (+/-0.45)	1.59 (+/-0.45	20.88 (+/-6.05)
K	4.96 (+/-0.19)	5.07 (+/-0.98)	4.11 (+/-0.53)	1.62 (+/-0.6)	20.29 (+/-8.15)

There were five breeds identified by the farmer classifications (Table 4.1) with no significant differences in milk production parameters or ECM over the entire lactation between breeds (Figure 4.2, $p>0.05$).

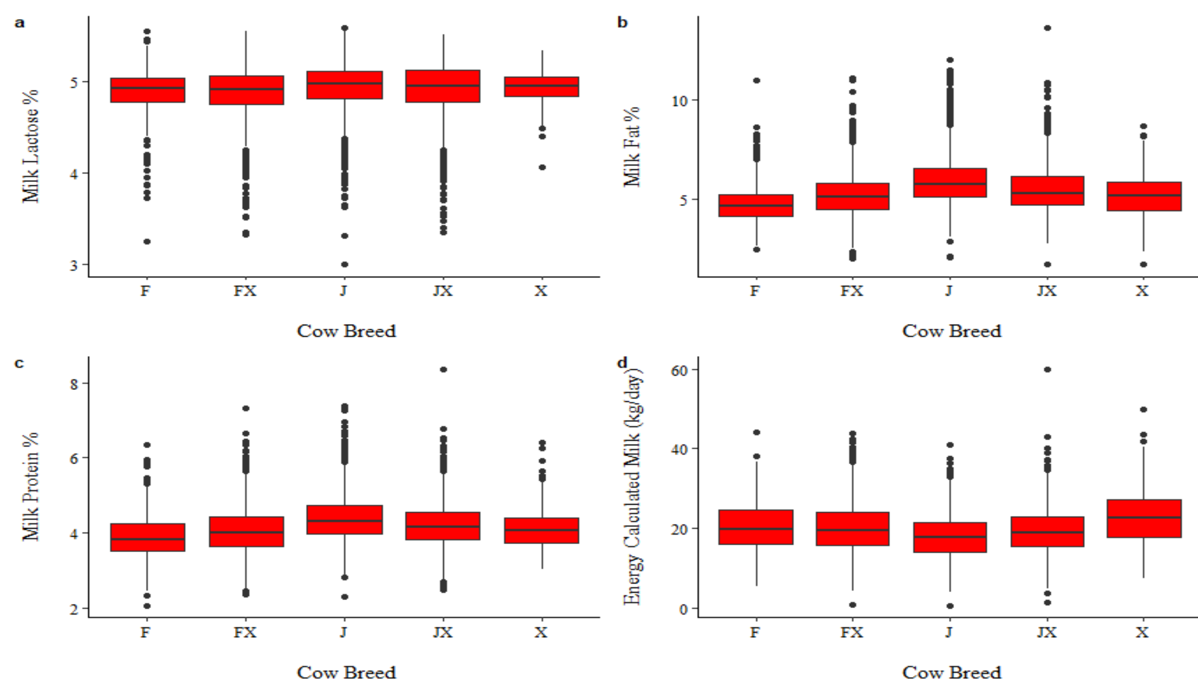

Figure 4.2 Cow breed and milk production indices a) lactose%, b) fat%, c) protein% d) ECM (kg/day).

The calculated ECM of each cow for all herd tests in each herd were similar (Figure 4.3a) with a trending increase with increasing cow age (Figure 4.3b). Although there was a marked difference in the mean and spread of IDEXX SP% between herds (Figure 4.3c), there appeared to be no biologically meaningful relationship between cow age (lactation) and IDEXX SP% (Figure 4.3d).

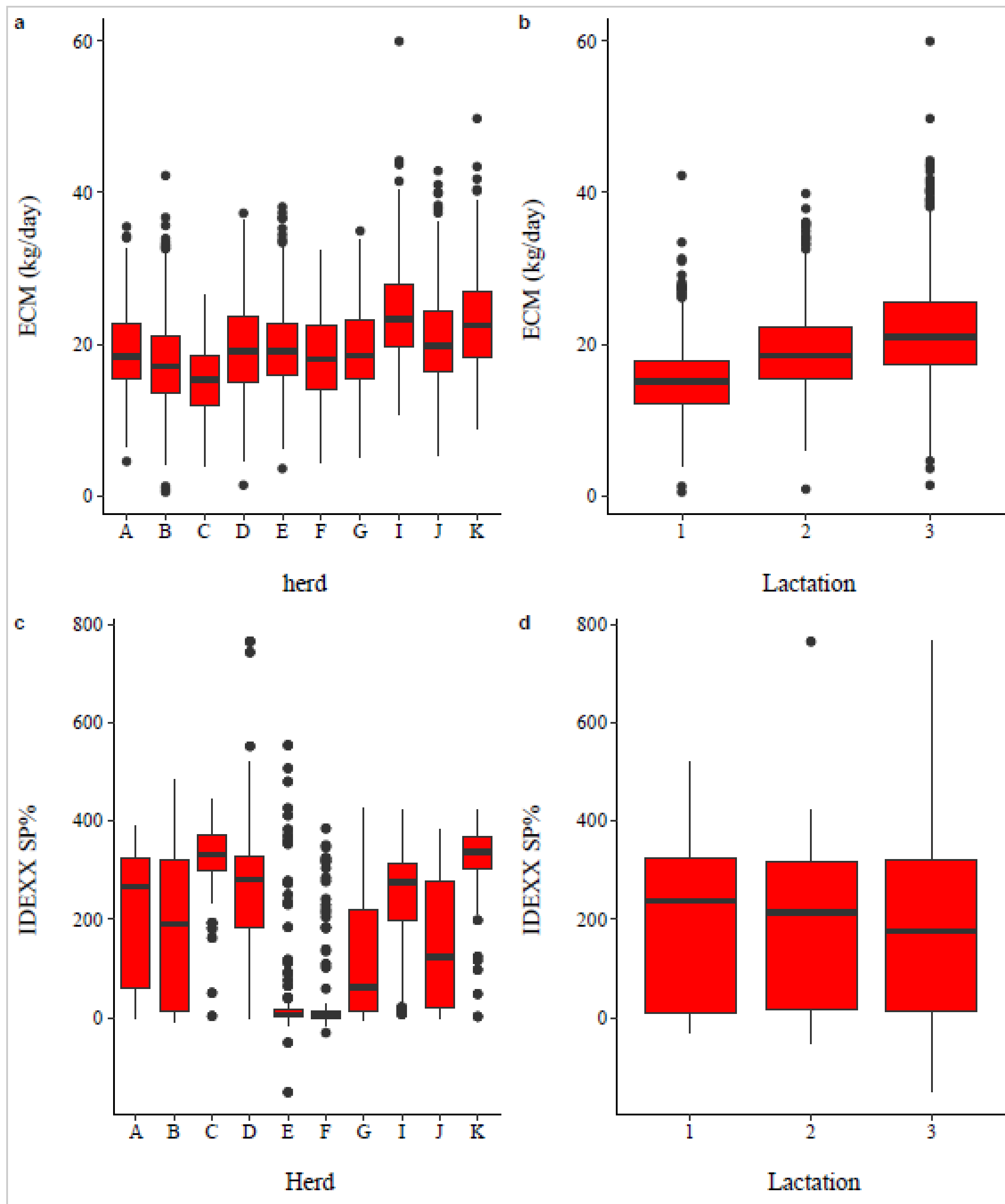

Figure 4.3 Box plots of (a) energy corrected milk (ECM) by herd, (b)ECM by the three lactation categories (first, second, third or more), (c) IDEXX SP% by herd, (d)IDEXX SP% by the three lactation categories (first, second, third or more).

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The milk production parameters from cows sampled in each herd and present at herd testing were plotted from the herd test results.

Total milk solids (MS) per cow per day declined over the lactation period for all herds (Figure 4.4). One herd (I) had more variable MS throughout the lactation. The greatest variation between herds was in both the early and later stages of lactation.

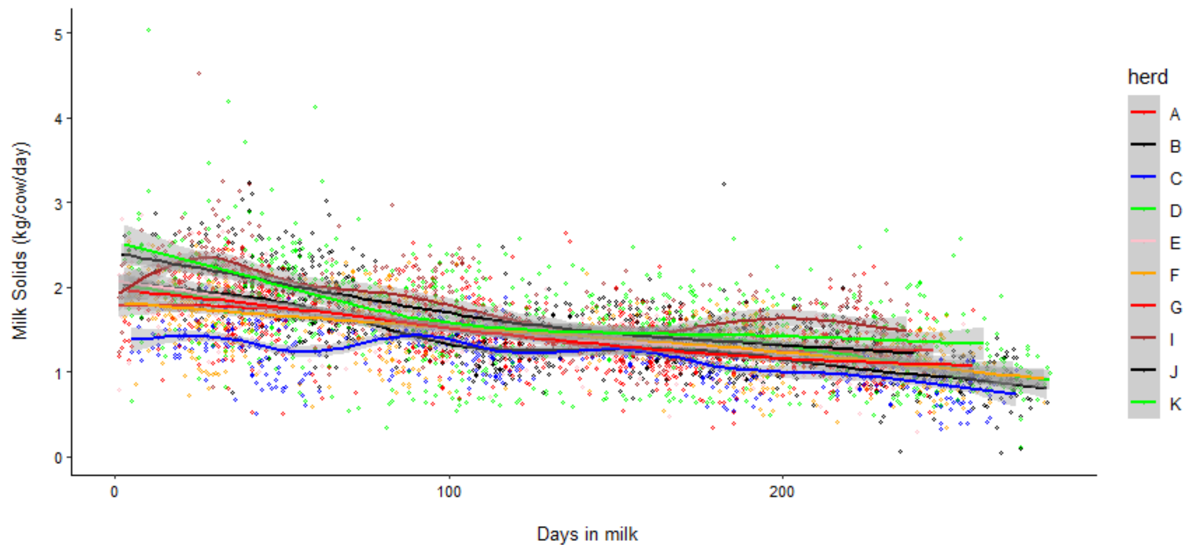

Figure 4.4 Milk Solids (kg/cow/day) production from each cow herd test over the length of the lactation with the mean result indicated by trend lines for each herd (herd).

Milk protein percentage (MP%) per cow per day declined in early lactation in all herds to then be stable until later lactation where MP% rose in all herds to varying degrees to end either similar or greater than at the start of lactation (Figure 4.5).

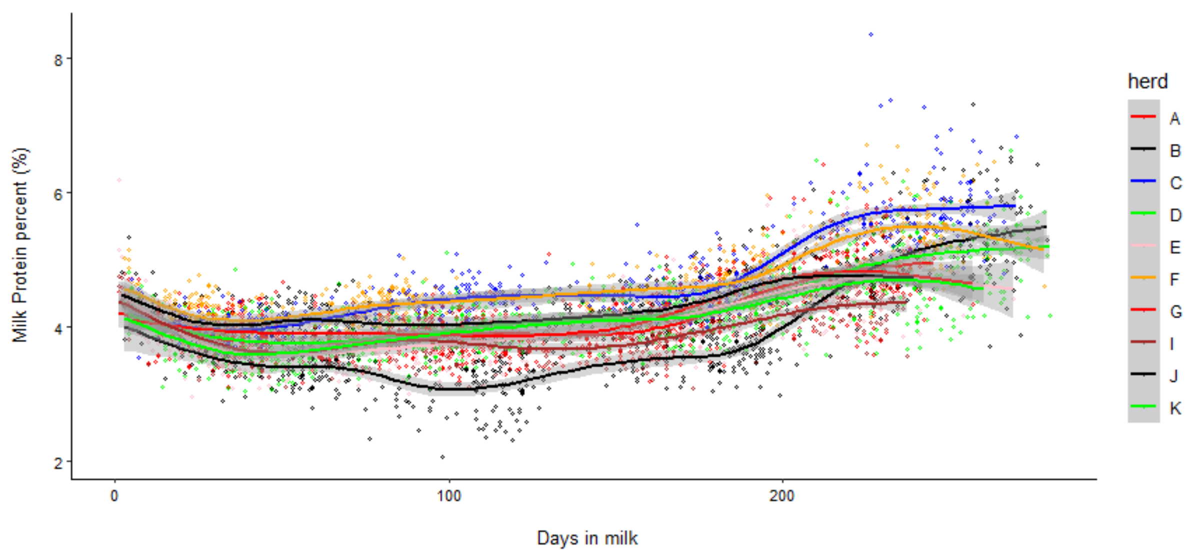

Figure 4.5 Milk protein percentage (%/cow/day) from each cow herd test over the length of the lactation with the mean result indicated by trend lines for each herd (herd).

Milk fat percentage (MF%) per cow per day was quite consistent between herds at the start of lactation to then become more variable while increasing toward the end of lactation (Figure 4.6).

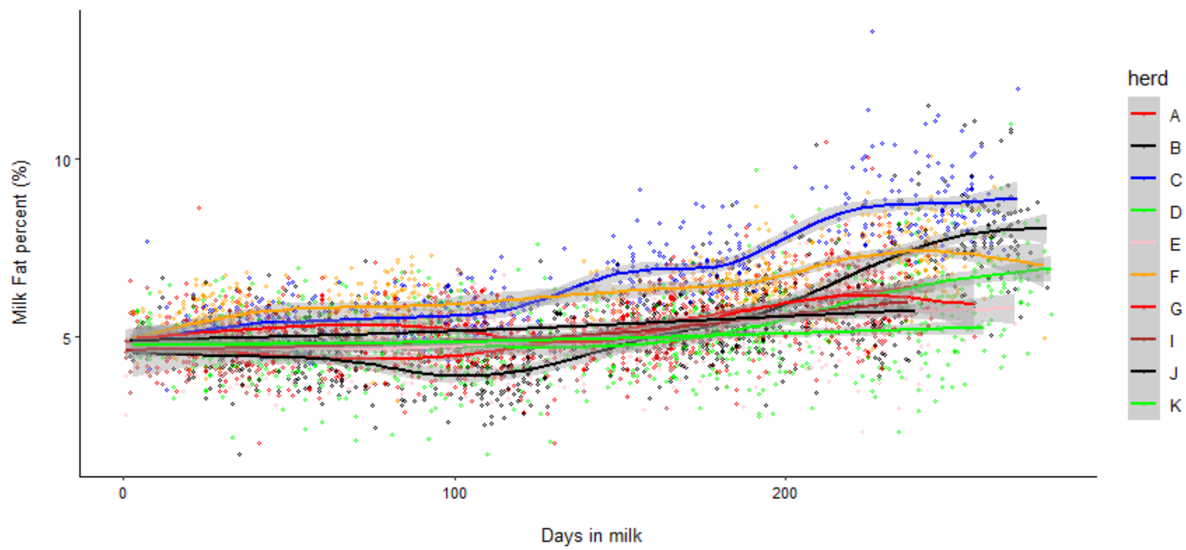

Figure 4.6 Milk fat percentage (%/cow/day) from each cow herd test over the length of the lactation with the mean result indicated by trend lines for each herd (herd).

Milk lactose percentage (ML%) per cow per day was relatively stable throughout the lactation for most herds except B (Figure 4.7). The ML% variability appeared less than that of MP% and MF%.

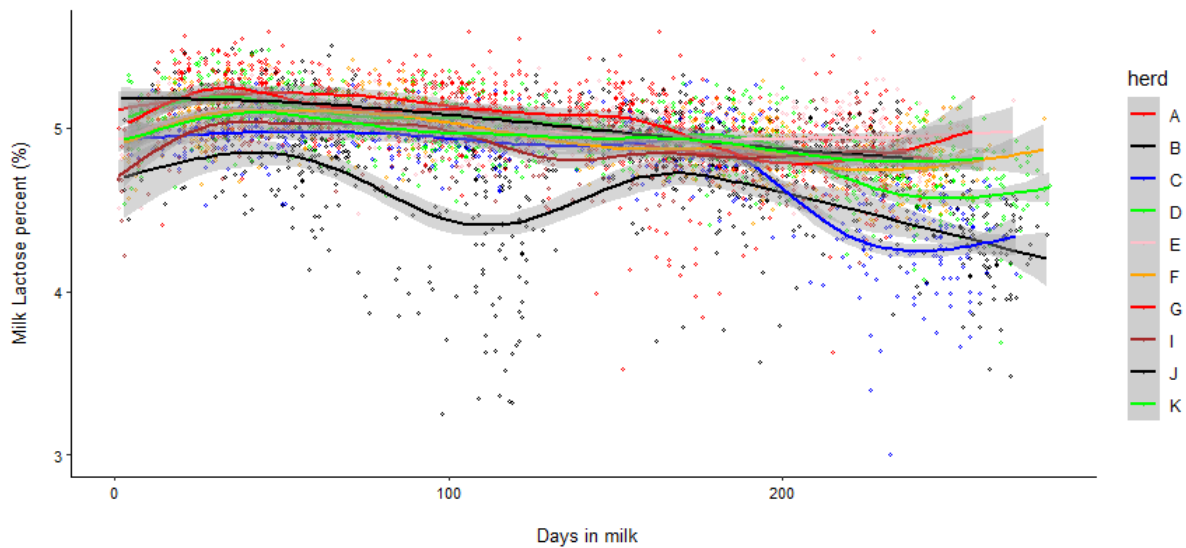

Figure 4.7 Milk lactose percentage (%/cow/day) from each cow herd test over the length of the lactation with the mean result indicated by trend lines for each herd (herd).

Energy Corrected Milk (ECM) kilograms per cow per day declined with increasing DIM (Figure 4.8)

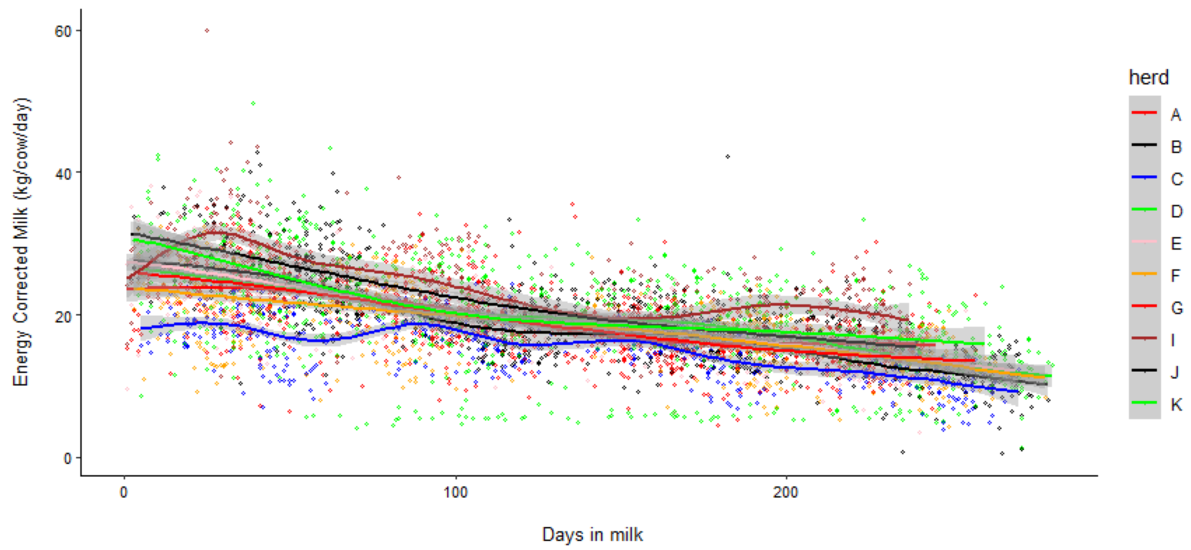

Figure 4.8 Energy Corrected Milk (kg/cow/day) from each cow herd test over the length of the lactation with the mean result indicated by trend lines for each herd (herd).

4.3.5 IDEXX antibody ELISA

IDEXX serum antibody ELISA was analysed on 1538 cows from the eleven herds representing 19-96% of the cows in each herd, and for 10/11 herds a BME was also analysed (Table 4.7). Herds were sampled in 2018 on April 10-12 (Herds F,A,B), April 24-26 (Herds D,H,G), May 7-9 (Herds C,I,E) and in 2021 on April 19 and 21 (Herds J and K). The percentage of cows in the *negative* diagnostic category ranged from 0-86%, *mild positive* 1 to 17%, positive 0-13% and *strong positive* 11-99%.

Table 4.7 Number and percentage of cows with an IDEXX serum analysis from each herd, the percentage in each IDEXX category, average SP% and associated IDEXX category and a summary for all cows sampled. The bulk milk ELISA category on the day of the serum sampling is also shown (one farm with no sample).

Herd	Number of cows sampled	% of herd sampled	IDEXX diagnostic category				Mean serum SP%	Mean serum category	BME category
			negative	mild positive	positive	strong positive			
A	135	21%	21%	8%	4%	66%	207	strong positive	no data
B	127	21%	29%	3%	13%	54%	184	strong positive	medium
C	129	100%	0%	1%	0%	99%	329	strong positive	strong
D	127	32%	10%	5%	8%	77%	256	strong positive	strong
E	147	29%	80%	5%	3%	12%	48	mild positive	low
F	159	96%	86%	1%	3%	11%	37	mild positive	no or very weak
G	149	91%	40%	17%	11%	32%	113	positive	strong
H	153	31%	4%	2%	3%	92%	300	strong positive	strong
I	112	19%	10%	5%	5%	79%	239	strong positive	strong
J	154	39%	34%	14%	9%	43%	139	positive	strong
K	146	29%	2%	1%	3%	95%	318	strong positive	strong
All farms	**1538**	**34%**	**30%**	**6%**	**6%**	**58%**	**197**	**strong positive**	**strong**

Herds E and F had the greatest percentage of *negative* cows (Table 4.7).

The weighted kappa between the IDEXX bulk milk ELISA diagnostic category and mean serum diagnostic category as determined by the mean serum SP% measured on the same day was 0.56 (95% CI 0.2-0.92; p=0.002) which indicates a *moderate agreement*.

The IDEXX antibody ELISA SP% had a bimodal distribution (Figure 4.9). with the quartiles defined in Table 4.8.

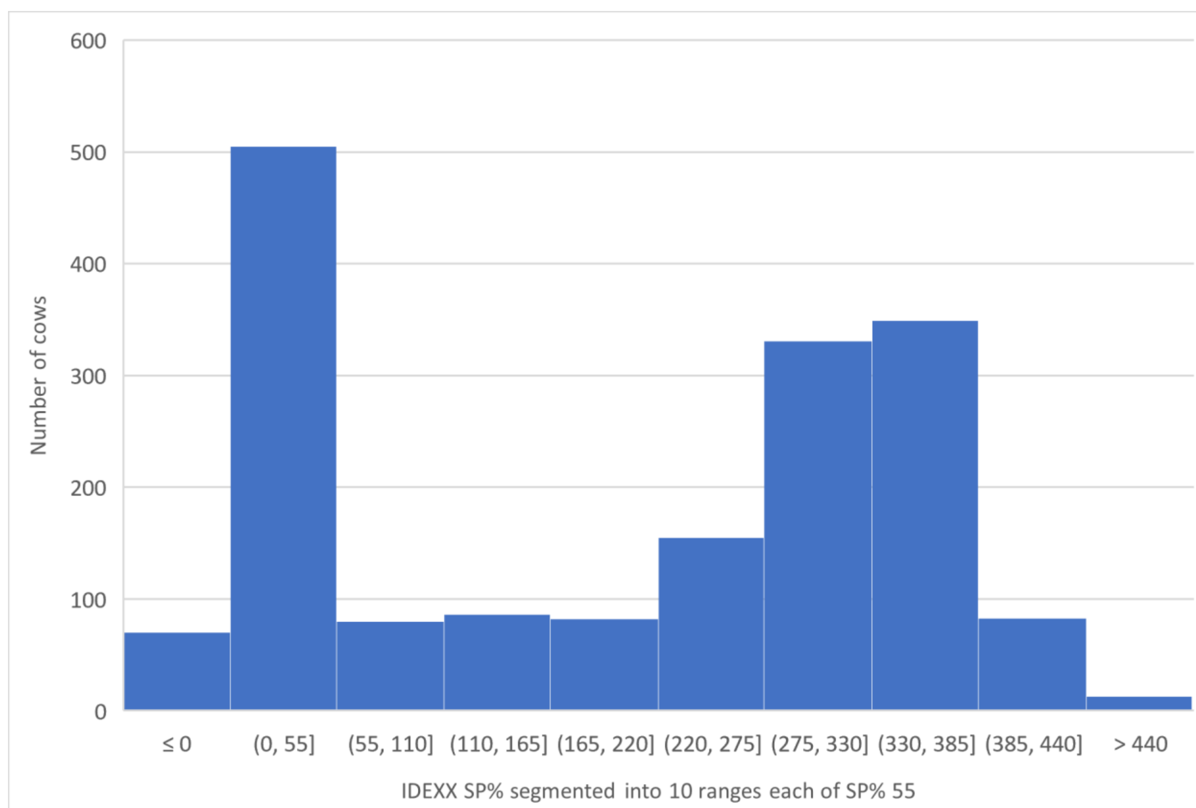

Figure 4.9 Histogram of the range of IDEXX antibody ELISA SP% from each cow (n=1754) from all 11 herds (SP% range -152 to 765) with bimodal distribution. Diagnostic cut point for positive categories > 30.

The IDEXX SP% results were divided into four quartiles for all herds for analysis with the parameters presented in Table 4.8, with all four diagnostic categories as determined by SP% being present in the second quartile.

Table 4.8 IDEXX SP% quartiles for all herds.

Quartile	First	Second	Third	Fourth
IDEXX SP%	SP% ≤10	10< SP% ≤ 190	190 < SP% < 317	SP% ≥317

In addition, in five herds a random selection of rising two-year-old heifers that were about to enter the herd were also sampled and on one farm eight-month-old calves were sampled (Table 4.9). The percentage of *negative* heifers and calves ranged from 0-90%, *mild positive* 0 to 5%, *positive* 0-36% and *strong positive* 9-100%.

Table 4.9 Number and percentage of rising two-year old heifers (and calves) in each IDEXX serum ELISA category.

Herd and Age	**Negative**	**Mild positive**	**Positive**	**Strong Positive**
A heifers	0 (0%)	0 (0%)	0 (0%)	21 (100%)
C heifers	0 (0%)	0 (0%)	0 (0%)	20 (100%)
C calves	17 (81%)	1 (5%)	1 (5%)	2 (9%)
F heifers	19 (90%)	0 (0%)	0 (0%)	2 (10%)
G heifers	1 (5%)	0 (0%)	8 (36%)	13 (59%)
H heifers	0 (0%)	0 (0%)	0 (0%)	20 (100%)

(Percentages do not total 100 due to rounding)

4.3.6 In-House antibody ELISA

The In-House ELISA optical density ratio (ODR) was analysed in 1276 cows (Figure 4.10) with an ODR ≥42 indicating a positive result.

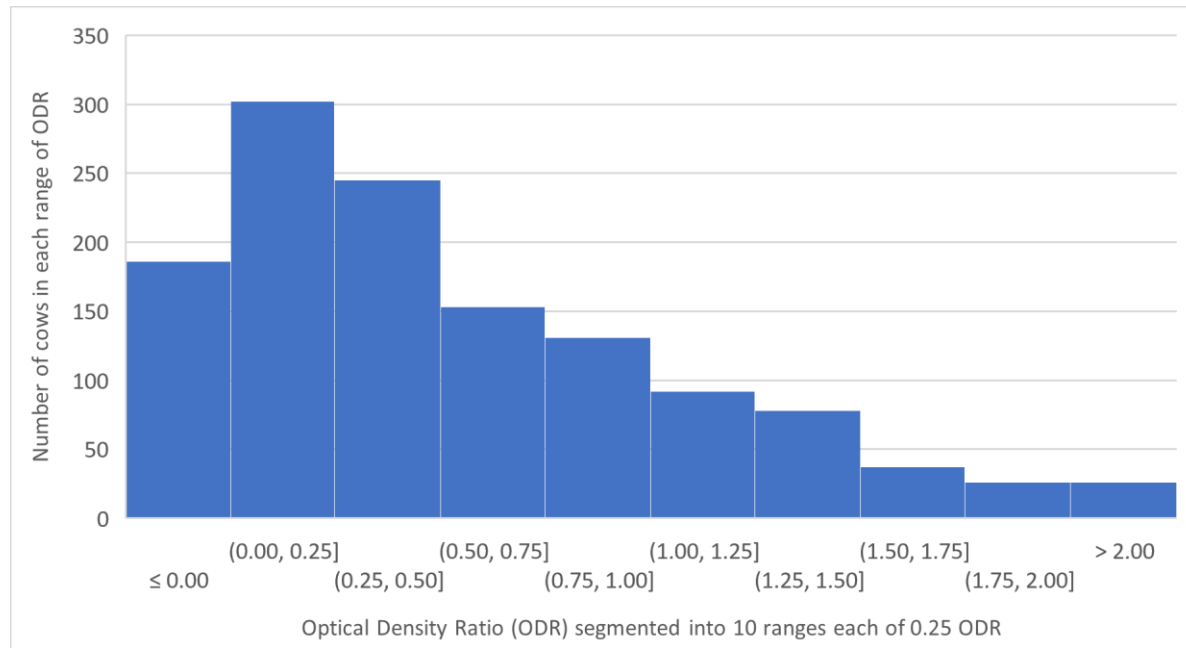

Figure 4.10 Histogram of In-House serum antibody ELISA ODR from 1276 cows.

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4.3.7 Concurrent analysis of cows from three herds coproantigen, faecal egg count and IDEXX antibody ELISA diagnostic tests

Coproantigen ELISA was completed on 311 samples from three Herds (A, J and K) and FEC were completed on 302 samples (Table 4.10).

Table 4. 10 Coproantigen and faecal egg count (FEC) results from three herds

Herd	A	J	K
Mean coproantigen value (range)	1.5 (-27.7 to 38.2)	2.7 (-21.1 to 106.6)	4.1 (-11.3 to 62.5)
Percentage coproantigen positive (Val >1.4)	26%	24%	42%
Mean FEC epg (range)	0 (0 - 0)	0.1 (0 - 2.2)	0.1 (0 - 0.8)
Percentage FEC positive	0	25%	18%
Paramphistome egg positive (percentage)	55/90 (61%)	106/117 (90%)	73/117 (63%)
Coinfection with liver and rumen fluke eggs	0/90 (0%)	28/117 (24%)	14/117 (12%)

The weighted kappa of 0.08 (-0.03 -0.18) p= 0.17 shows a *slight agreement* between coproantigen positive and faecal egg count (FEC) positive cows (Table 4.11). Removing Herd A in which all cows were FEC negative did not alter the relationship (weighted kappa = 0.07, p >0.28).

Table 4. 11 Table of faecal egg count and coproantigen values used for calculations of association.

All herds	Coproantigen negative	Coproantigen positive	Total
Faecal egg count negative	184	73	257
Faecal egg count positive	28	18	46
Total	212	91	303

The spread of Coproantigen values is represented by box plots (Figure 4.11), with Herd K have a higher mean value than Herds A and J with a value of >1.4 representing a positive result.

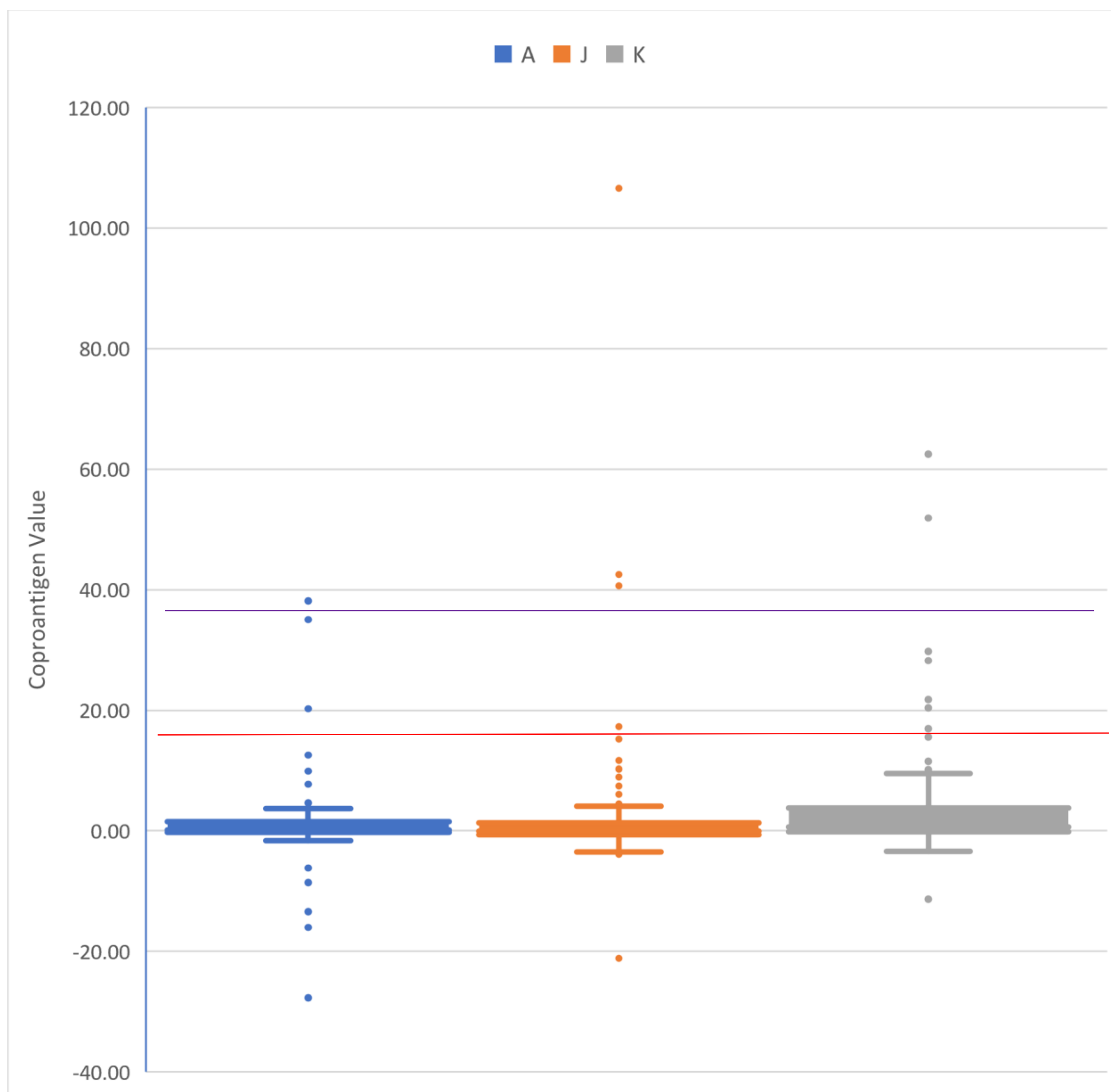

Figure 4.11 Box plots of coproantigen ELISA value of cows sampled from three herds (A, J and K). The red horizontal line indicating coproantigen value ≥17.5 indicative of a fluke burden of 10 or more flukes with the purple horizontal line indicating coproantigen value ≥37 indicative of a fluke burden of 30 or more flukes.

The spread of IDEXX SP% is represented by box plots (Figure 4.12) with Herd K having the most compact and highest results.

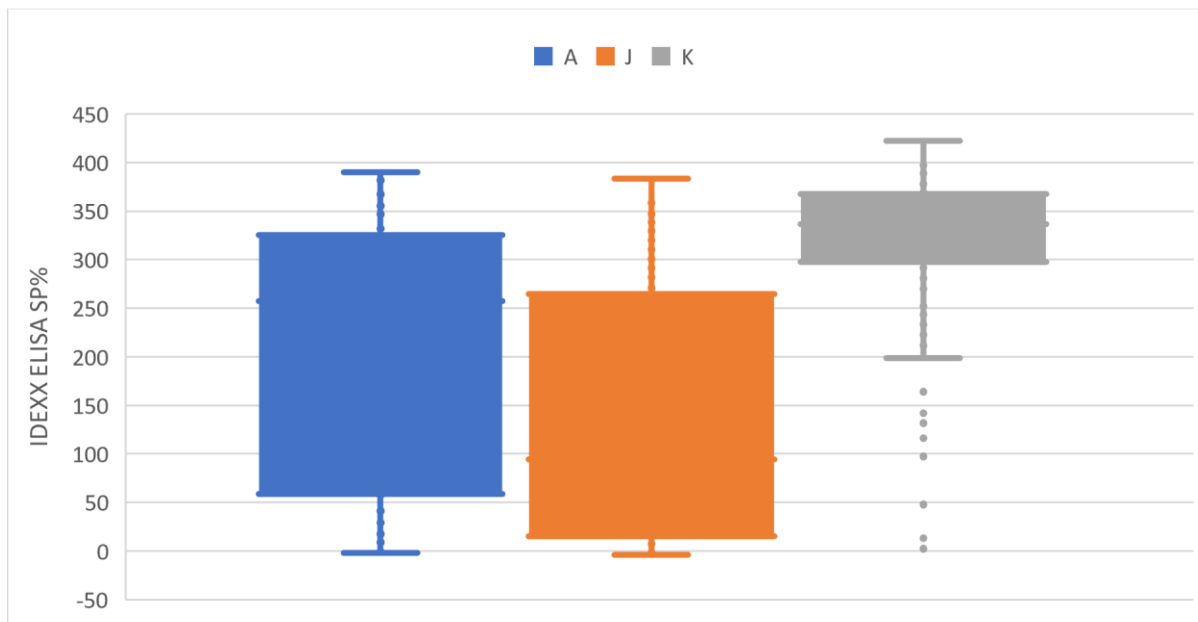

Figure 4.12 Box plots of IDEXX serum ELISA SP% from cows sampled from three herds (A,J and K).

The spread of faecal egg counts (FEC) is represented by box plots (Figure 4.13) with Herd J having the highest results even though all herds had low FEC recorded.

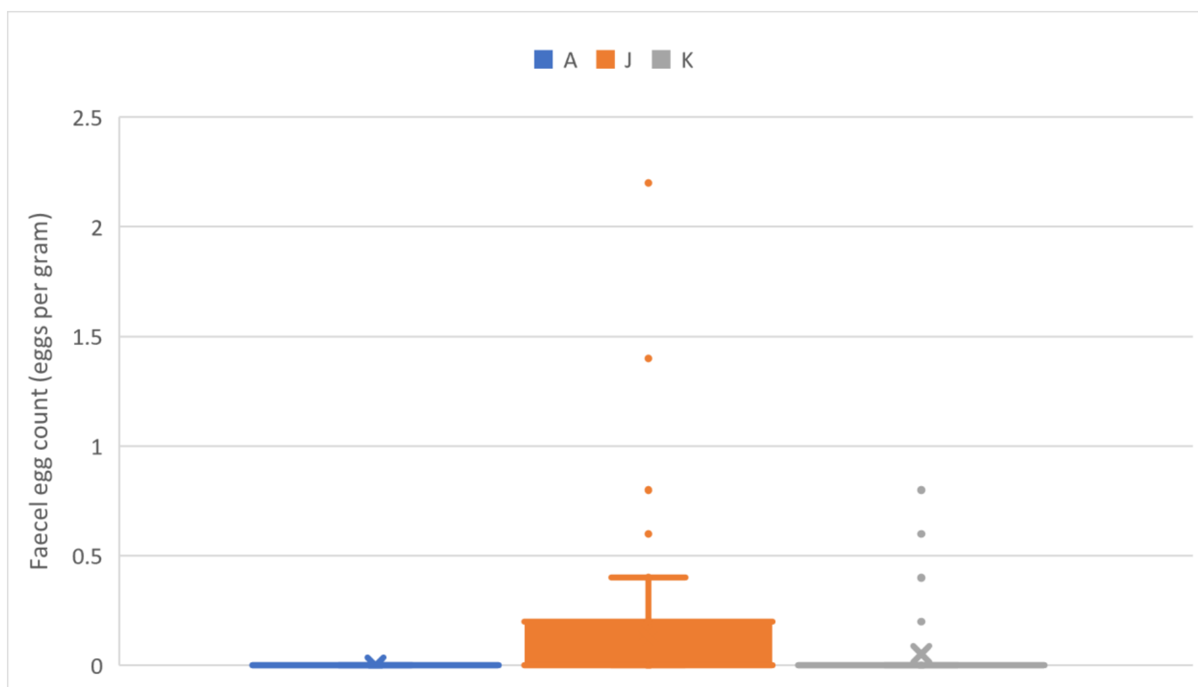

Figure 4.13 Box plots of faecal egg counts (eggs per gram of faeces) of cows sampled from three herds.

4.3.8 Intraclass correlation

The intraclass correlation (ICC) of the serum IDEXX serum SP% calculated the proportion of variance at the cow level of 59%, between herds 40% and residual variance of 1%.

4.3.9 Association between cow breed and milk production parameters

The only association determined was Jersey cows being associated with higher MF% (p=0.04).

4.3.10 Association between antibody ELISA tests and milk production parameters

One cow was removed from the analysis as the ODR was 10 times lower than the next lowest reading and was identified as a likely input error. The only significant relationship (p<0.05) was for Milk Fat percentage (MF%) which decreased with increasing IDEXX SP% (Table 4.12). This was not observed with the IH ELISA values.

Table 4.12 The level of significance (p values) of antibody detection ELISAs (In-House ELISA optical density ratio and quartiles IDEXX ELISA SP%, log In-House ODR, IDEXX SP% and quartiles, IDEXX diagnostic category (negative, mild positive, positive, strong positive) when compared to milk production parameters.

P values for variables for antibody detection ELISA assays					
	IH ELISA	IH Quartiles	IDEXX SP%	IDEXX SP Quartile	IDEXX category
Protein %	0.25	0.12	0.54	0.17	0.23
Fat%	0.06	0.02	0.004	0.13	0.34
Lactose %	0.94	0.91	0.78	0.89	0.54
MS	0.087	0.22	0.42	0.34	0.52
ECM	0.079	0.16	0.37	0.36	0.51

There is a near-linear relationship between IDEXX SP% and milk fat % (MF%) in all herds. The final model for the effect of SP% on MF% showed a significant effect of breed ($p<0.0001$) but not for lactation ($p=0.06$). There was strong evidence for an effect of SP% on MF%, with the % MF decreasing 0.0004 (95% CI 0.0001—0.0007) for every unit increase in IDEXX SP% ($p=0.003$).

The estimated MF% for each cut point for the IDEXX categories of herd prevalence, at mild positive (SP% =30) the predicted MF% was 5.2% (95% CI 4.9 —5.5), at positive (SP% = 80) the predicted MF% was 5.18% (95% CI 4.88—5.48) and at strong positive (SP% = 150) the predicted MF% was 5.15% (95% CI 4.85—5.45).

Model Summary

Parameter	Coefficient	SE	95% CI	t(4950)	p
Fixed Effects					
(Intercept)	4.80	0.16	(4.48, 5.11)	29.89	< .001
SP	-3.92e-04	1.30e-04	(-6.47e-04, -1.37e-04)	-3.01	0.003
DIM	0.50	0.11	(0.29, 0.71)	4.66	< .001
Breed (FX)	0.24	0.06	(0.13, 0.36)	4.21	< .001
Breed (J)	0.60	0.07	(0.48, 0.73)	9.24	< .001
Breed (JX)	0.45	0.06	(0.33, 0.56)	7.64	< .001
Breed (X)	0.78	0.13	(0.52, 1.03)	5.95	< .001
DIM2	0.31	0.01	(0.29, 0.33)	28.77	< .001
DIM3	0.07	0.01	(0.04, 0.09)	5.61	< .001
Random Effects					
SD (Intercept: cow:herd)	0.53				
SD (Intercept: herd)	0.47				
SD (DIM: cow:herd)	0.22				
SD (DIM: herd)	0.33				
Cor (Intercept~DIM: cow:herd)	0.80				
Cor (Intercept~DIM: herd)	0.68				
SD (Residual)	0.59				

Model: Fat. ~ SP + DIM + Breed + DIM2 + DIM3 (4966 Observations)
Residual standard deviation: 0.591 (df = 4950)
Conditional R2: 0.772; Marginal R2: 0.341

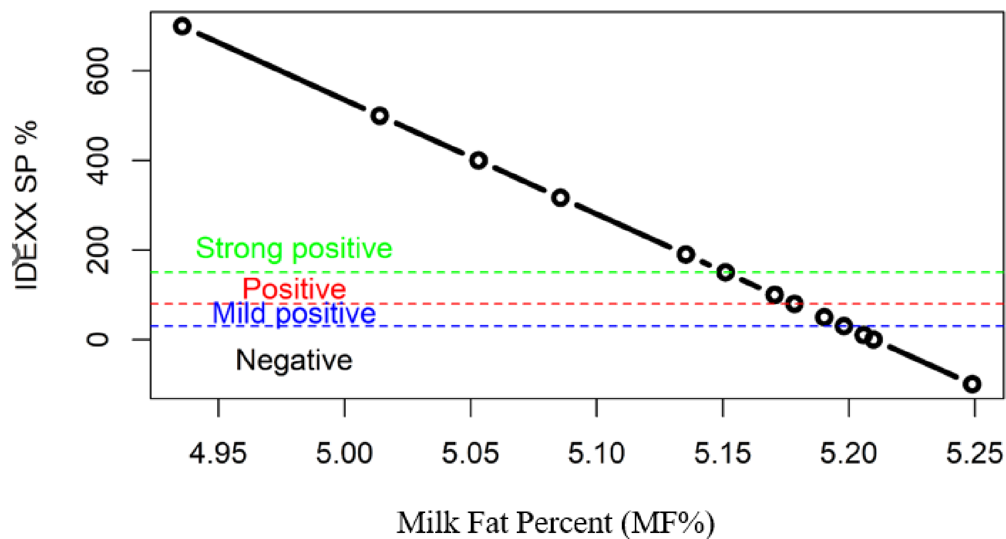

Figure 4.14 The relationship between Milk Fat Percentage (MF%) and IDEXX SP%, with Milk Fat Percentage being greatest when IDEXX SP% is low. The manufacturers cut points for different categories of IDEXX SP% are shown on this graph.

4.3.12 Association between faecal analysis and milk production parameters

There were no associations between FEC and milk parameters (Table 4.13).

Table 4.13 The level of significance (p values) of diagnostic test variables (coproantigen value, coproantigen diagnostic category, faecal egg count) when compared to milk production parameters.

P values for variables for Coproantigen ELISA and faecal egg count			
	Coproantigen ELISA Value	Coproantigen category (positive / negative)	Faecal egg count (epg)
Protein %	0.60	0.73	0.11
Fat%	0.66	0.7	0.1
Lactose %	0.34	0.9	0.2
MS	0.44	0.82	0.74
ECM	0.45	0.78	0.63

4.4 Discussion

This study confirms the findings of Chapter 3, that liver fluke is indeed a common parasitic infection of dairy cows in some herds on the West Coast as identified by serum antibody ELISA, with marked variation both within and between herds. The variability of the serum antibody reaction within a herd was evident in the adult cattle and was also apparent in the heifer and calf samples (Table 4.9) which did not always align with the results from the lactating cows on the same farm, likely being related to grazing different areas of the farm. The diagnostic categories defined by the IDEXX bulk milk and serum ELISAs showed only a *moderate* relationship (weighted kappa 0.56), indicating that the prediction of the liver fluke prevalence in each herd using the two tests are relatable but not especially strongly.

There was a significant relationship between IDEXX SP% and milk fat % (MF%) in all herds with every increase in SP% resulting in a MF% decrease of 0.0004% (95% CI -0.0006—0.0001) (p=0.004). however, there was no significant relationship between IDEXX SP% and any other milk parameter.

The presence of fluke in both young and adult cattle and high percentage of cows with positive IDEXX result implies that cattle on the West Coast are susceptible to repeated infection with immature flukes and the presence of the adults in the bile ducts that may result in choloangiohepatic pathology and fibrosis so severe that cattle do not benefit from treatment to remove the flukes as has been observed elsewhere (Charlier *et al.* 2012). These repeated infections resulting in cumulative liver damage may explain why older cows (lactation 3+) were not associated with a greater milk production impact based on infection in this lactation. Younger cattle were preferentially sampled in this study in an attempt to reduce any impact from fluke infections in previous years.

4.4.1 Herd testing frequency and days in milk at each herd test

The cows in the study had milk production monitored three to four times over the period of the lactation, common practice in New Zealand dairy herds and fewer than overseas studies where daily, 4-weekly or 6-weekly monitoring occurs (Charlier *et al.* 2012; Kostenberger *et al.* 2017; May *et al.* 2020). This infrequency of monitoring weakens the dataset, making it is more difficult to observe trends with fewer datapoints. The components of milk change throughout the lactation, in general fat and protein yield (kg) both increase in early lactation and then decline whereas fat % and protein % are high at the start of lactation, decrease as the lactation progresses to increase again in the latter stages although they finish lower than the start (Silvestre *et al.* 2009). Fat and protein % both increased through lactation in this study. Milk lactose yield and % were also measured in this study with lactose % decreasing through the lactation.

The stage of lactation is often not referenced in published papers, perhaps due to the constant movement of cows into and out of the lactating herd which is more common in overseas farm systems. In spite of all herds calving in the spring there is still a considerable range of DIM at each herd test of 78, 155, 152 and 129 indicating there is still a large spread of calving dates (Figure 4.10).

4.4.2 IDEXX ELISA

The distribution of IDEXX SP% and diagnostic categories shows most cows being either *negative* or *strong positive* (Table 4.4, Figure 4.5) making analysis of the diagnostic categories difficult, other than a comparison of the two extremes. These cattle were all sampled in the autumn near the end of that lactation. Infection may have occurred at any time since the last anthelmintic treatment, if any were given. As cows cannot be treated whilst lactating this was at least during the previous dry period (drenching history of the herds was not determined). Liver fluke may be long lived in cattle but it is agreed that 75% survive five to twenty-one months (Ross 1968; See Section 1.4.8) with increased longevity possibly linked to lower numbers of flukes present (Takeuchi-Storm *et al.* 2018). Based on low FEC and coproantigen values found in the three herds sampled it is likely only low numbers of flukes were present so this increased longevity may be a feature of the herds in this study. Also, it was shown in Chapter 3 that fluke infection can probably occur over the winter and early spring so it is plausible that cows can become infected throughout the year. In this study it is not possible to determine the dynamics of fluke infection including

1. Time of first infection.
2. Total number of metacercariae ingested.
3. Temporality of metacercarial ingestion.

4. The proportion of immature and mature flukes present at the time of testing.

5. The effect of self-cure.

As can be seen in the results the IDEXX test, the antibody reaction of individual cows to liver fluke infection varies widely both within and between herds. In a study of 11 herds in Flanders, Belgium (Charlier *et al.* 2012) where the geography is relatively constant, there was little variation between herds which contrasts with this study. In Chapter 3 of this thesis, great regional variation of levels of infection were noted and it was suggested that climatic and environmental factors that favour the maintenance of the intermediate host were a cause. The geography of the West Coast varies greatly from free draining alluvial flats to "humped and hollowed" waterlogged flats. This feature of great local variation is more than is experienced in most other dairy farming regions in New Zealand. In that same Belgian study, 51% of the total variance was at the cow level which is similar to the 59% in this study, both indicating significant differences in the antibody response of animals that would be thought to be exposed to similar infection factors. Some of the within herd variability could be due to the practice of separating the larger herds into two or more groups for ease of management which could result in one group grazing part of the farm with a greater risk of fluke infection. This is evident in Herd C with 100% of heifers with a *strong positive* result and 81% of calves, which are one year younger, having a *negative* result yet grazing on the same farm (Table 6.6). The immune response that is detected by the assay is also complex, peaking 8 weeks post infection (wpi) when naïve cattle were infected (Reichel 2002) whereas in naturally infected cattle the median SP% doubled from 123 to 283 over 90 days (Munita 2019) moving from the *positive* to *strong positive* category. One of the aims of that study was to assess the assays after anthelmintic treatment so it is assumed the cattle were moved to fluke-free pasture after treatment so the change in SP% was due to the maturation of the current fluke burden and maturity of the immune response in the cow.

It is difficult to accept that so few cows would be in the *mild positive* and *positive* diagnostic categories, suggesting that antibody ELISA assays have narrow cut points between diagnostic categories which can lead to overinterpretation of the results. In some studies associations with milk production parameters have been found when using the quartiles of the ELISA assay used.

The results from the coproantigen study and faecal egg counts indicate that only low burdens of fluke were present in infected cows yet they were demonstrating a large percentage of *strong positive* ELISA values.

The bulk milk tank ELISA from milk collected on the day of sampling was mostly in agreement with the mean SP% of the cows that were serum sampled with both indicating a high percentage of cows with a *strong* antibody response to liver fluke infection (Table 4.4) indicating that the bulk milk result is consistent with the individual cow samples and hence a reliable way to assess the herd.

The In-House ELISA ODR distribution has a single modal distribution with a right shift and fewer cows having a positive result. Although these two ELISA are detecting IgG antibodies to ESA, the IDEXX test uses a refined antigen. These results further illustrate the poor test characteristics of this In-House assay. This is also seen in that the In-House ELISA did not show a significant effect on MF% whereas the IDEXX ELISA did.

A weakness of this current study is that the analysis of the impact of liver fluke infection over an entire lactation is only assessed by a single determination of infection status near the end of the lactation.

There is no indication of the temporality, severity or maturity of infection of individual cows during the lactation (Hutchinson 2003; Munita 2019).

4.4.3 Coproantigen and faecal egg counts and IDEXX ELISA in three herds.

The mean coproantigen percentage positivity (value) of the three herds in this study are similar to those found by Novobilsky (2015) investigating closantel inefficacy on three beef farms in Sweden, but lower than the cattle sampled in Chapter 2 of this thesis by a factor of 5.7 which is again lower than that found in dairy cattle in Australia by a factor of 1.7 (Brockwell *et al.* 2013; Elliott *et al.* 2015). These results are consistent with low burdens of liver fluke being present in these cows. In Chapter 2 there was a relationship between coproantigen value and natural log of the total fluke counts with R^2 = 0.61 with a curvilinear relationship. Infection with ≥10 flukes (Charlier *et al.* 2008) or ≥30 flukes (Vercruysse and Claerebout 2001) are considered to be production limiting. In the current study only 14/311 (5%) cows have a coproantigen value ≥17.5 indicating they are likely to have ≥10 flukes and only 6/311 (2%) have a coproantigen value ≥37 indicating they are likely to have ≥30 flukes. This indicates that although infections appear common, the number of flukes is low.

The weighted kappa of only 0.08 (95% CI -0.03-0.18; p= 0.17), demonstrates there was only a *slight agreement* between coproantigen positive and FEC positive cows. The mean FEC in this study was low at 0.1 eggs per gram (epg; Table 6.6), which is even lower than was seen in Chapter 2 (0.3epg). Nevertheless, this again illustrates the low predictive value in using FEC in cattle with low fluke burdens especially. Interestingly, the IDEXX SP% on these three herds were 141, 139 and 318 compared with 247 in Chapter 2 which are all in the *strong positive* category. This again reflects the limited value that can be placed on antibody serology figures to estimate fluke burdens. This is consistent with infection being common even though burdens are low.

Egg output from flukes is maximal 3-8 months post infection, then reducing and also being undetectable in cattle 10 months and more post-infection (Ross 1968) which may be occurring in the sampled herds. The low FEC (Table 4.10) decreases the sensitivity of the test (Kelley *et al.* 2021a). A faecal egg count of >5 epg is considered to be indicative of a production limiting infection (Malone and Craig 1990; Vercruysse and Claerebout 2001) with no cows in this study reaching this threshold. A positive faecal egg count in a 4g sample is 10.7 times more likely to be indicative of a production limiting infection with liver flukes rather than it is free of infection or has a low fluke burden (Charlier *et al.* 2008). Thus, using this interpretation and as suggested above, there is inconsistency between the egg count evaluation and coproantigen values. For the latter at least some were indicating infections high enough to be production limiting. Interestingly, the coproantigen positivity rate and IDEXX SP% are both higher in Herd K but the percentage of cows FEC positive are similar, further complicating the interpretation of diagnostic tests.

These results highlight the diagnostic uncertainties in herds with a high infection prevalence but a presumed low fluke burden per cow. While it is possible to be confident that flukes are present in these herds it is not simple to determine if it is at a level that would be production limiting and thus if milk production would improve after treatment of infected cows. Presumably these three herds are representative of the others in this study and herds on the West Coast in general. This finding supports the use of diagnostic tests in parallel if only one sampling point is available (George *et al.* 2019) or

serial sampling (Sekiya 2013) rather than a single test to improve the clinicians ability to translate the tests results for the herd owner to make informed management decisions.

4.4.4 Milk production

Milk solids (MS) production per cow per day are expected to peak 42 to 64 days into lactation and then decline as lactation progresses (Figure 4.3). This peak and decline is only evident in one herd with the remainder showing a steady decline with some fluctuation and is likely to be due to cows not being evaluated until a mean of 35 days into lactation with a range of 1-79 days (Table 11). The mean herd production as represented by the trend line for each herd indicates a maximum MS/cow ranged from 1.4 to 2.6 kgMS/cow with the New Zealand peak average on 2018/19 lactation (the one subsequent to this data collection) was 1.99 kgMS/cow and for the West Coast and top of the south was 1.96 kgMS/cow (Latest DairyBase benchmarks - DairyNZ). The MS production from the herds in this study appear to be consistent with what would be expected for a herd in this region.

The calculation of energy corrected milk (ECM) for all herd tests was similar to each other (Figure 4.2a) indicating that any negative influence may be moderated by the adequate provision of other factors impacting milk production, with variation within herds similar between herds. Chronic liver damage and liver fluke infection in the previous lactation have been shown to impact milk production (Charlier *et al.* 2012) and the finding of 58% of cows with a *strong positive* result indicates they are also likely to be reinfected each year, resulting in chronic liver pathology. No effort was made to determine the degree of liver damage in cows in this study through serum biochemistry due to cost constraints, but this information could have been useful. In one study, cows in the upper quartile of a serum antibody test results did not increase milk production after anthelmintic treatment indicating that liver pathology was too marked (Charlier *et al.* 2012).

The interaction of BME IDEXX SP% and MF% has been shown in other antibody ELISA studies comparing the upper against the lower quartile, or *strong positive* to *negative* with a decrease of 0.06kg/cow/day (p<0.001;(May *et al.* 2020)), 0.091 (p=0.004;(Kostenberger *et al.* 2017)) using the Svanovir ELISA, and 0.06 (p<.001;(Charlier *et al.* 2007)) using a crude antigen ELISA. In the present study the relationship between IDEXX ELISA SP% and MF% is linear, with a high p value (p=0.004). However, the R^2 of 0.31 indicates the line was not as linear as it visually appears. This lower R^2 may be in part due to MF% being more variable particularly toward the end of lactation (Figure 4.5). For every increase of IDEXX SP% the MF% decreased by 0.0004. While these changes may appear minor, it must be remembered that the mean MF% was 5.51 with a range of 1.7-13.6%. The MF% of a cow at the lower end of the *strong positive* diagnostic category (SP% 150) would be 0.048 MF% points lower than that of a cow at the upper level of a *negative* diagnostic category (SP% 29) which is potentially an important change. Given that some cows had both lower and higher SP% values than these cut points the difference for at least some cows will be greater than this. This is compared to the similar trend in MF% seen in the following study in Chapter 5 (Section 5.4.3).

Only one small study which investigated the association between milk lactose and liver fluke infection where cows were monitored for five days after liver fluke treatment and no association was noted (Takata *et al.* 1980), a finding also reflected in this present study.

4.4.5 Paramphistome Infection

Eggs of the rumen fluke paramphistome parasite (*Calicophoron calicophorum*) were present in 61, 63 and 90% of faecal samples from three herds (Table 4.10). One farm had no cows with fluke eggs present but 61% with paramphistome eggs with the other farms having 25 and 90% and 18 and 63% (Table 4.10). Coinfection with the two parasites was present in 0, 18 and 23% of cows.

Co-infection of these two parasites is also noted in international studies (Kajugu *et al.* 2015; Jones *et al.* 2017; Ploeger *et al.* 2017; O'Shaughnessy *et al.* 2018; May *et al.* 2019; Ico-Gomez *et al.* 2021; Kelley *et al.* 2021b; Delafosse 2022) with the presence of the adult parasite not considered to be of clinical importance (Delafosse 2022). The most probable intermediate host snail for *Calicophoron calicophorum* in New Zealand is the flat spiralled planorbid snail *Planorbis kahuika* (Jonathan 1950; Charleston 1997). In some countries these two parasites share intermediate hosts so may compete to infect them whereas in New Zealand they utilise separate intermediate host snails (Jonathan 1950; Charleston 1997) although the development of PCR testing of snails would be useful to determine if this is the current situation. The finding of these two trematode parasites coexisting on these farms is of scientific interest and as *P. columella* has not yet been identified on the West Coast, a survey of the presence of the parasite and infection with either of these parasites is warranted.

4.4.6 Future Study

Future study would likely make greater use of coproantigen testing to get a more accurate evaluation of the infection level in individual cows with less use of serology. These current results indicate that many cows have high circulating IgG antibodies indicating past and possibly present fluke infections but the only relationship with milk production was a decrease of milk fat percentage as IDEXX SP% increased. Further study would assess the antibody titre of cows earlier in lactation as well as near the end to investigate any interaction between these and milk production parameters.

4.5 Ethics permission

This study was performed under the approval of Massey University Animal Ethics Committee, Protocol MUAEC 18/13

4.6 Supplementary Materials

Supplementary materials for Chapter 4 are contained in Section 3.2.4.

Chapter 5: Longitudinal Study of the impact of the change in liver fluke infection status between spring and autumn on milk production over the course of one lactation on four farms on the West Coast of the South Island.

5.1 Introduction

In Chapter 4 "Cross Sectional Study", cows from eleven herds were serum sampled near the end of the lactation to investigate the impact of anti-fasciola antibody concentration on milk production in that lactation. A weakness of this study was that the timing of infection could not be determined and this may be important when detecting small changes in milk production. It is accepted that both the migration of juvenile flukes through the liver parenchyma (Dawes 1963a; Wilson *et al.* 1998) causing considerable damage and fibrosis to liver parenchyma as well as the mature flukes causing haemorrhage and ulceration of the bile ducts (Ross 1966; Behm and Sangster 1999) can both contribute to production losses.

The antibody response of a cow to liver fluke infection is non-linear with antibodies detected by the IDEXX test two weeks post infection (wpi) reaching a maximum SP% eight wpi (Reichel 2002). To further complicate matters, in a study of naturally infected Irish cows the SP% more than doubled from 123 to 283 over the 90 days of the study, moving from the *positive* to *strong positive* diagnostic category (Munita 2019) even though the animals were grazing fluke free pastures during that period. Antibody decay also needs to be considered for the dynamics of the titre detected, so with these and likely other factors involved, serial sampling of animals may provide more information compared to a single test point.

The gross liver pathology score of adult cows was greater than the younger steers in Chapter 2 and this was not related to the current fluke burden. The calcification of bile ducts as a result of inflammatory response to the residence of adult flukes was severe in some livers. More severe gross liver pathology in a dairy cow as a result of fluke infections over the years may impact milk production regardless of current infection, so sampling younger cattle may be a better indication of milk production losses due to current infection.

Chapter 3 highlighted regional variation in liver fluke infection in herds as determined by a bulk milk ELISA test. Within those regions there were herds that were consistently a *no or very weak* diagnostic category as well as those that were reinfected by October (spring) to have an antibody concentration in the same diagnostic category as in March (autumn) including those where a liver fluke specific anthelmintic treatment had been administered.

The aim of this longitudinal study was to serum sample younger cows from four herds on two occasions during the lactation, once in November (spring) and again in March (autumn) to investigate the associations between the change of anti-fasciola antibody concentration at those two time points with milk production parameters. This then allows the economic cost of any loss of production to be estimated.

A supplementary aim was to assess the test characteristics of the In-House ELISA using three datasets, including some from this study, using the IDEXX ELISA as a reference standard.

5.2 Materials and Methods

5.2.1 Study design

A longitudinal study was conducted where up to 150 cows from each of 4 herds were serum sampled during the same week in November 2018 (spring) and again in the same week in March 2019 (autumn). Serum samples were analysed for the presence of anti-fasciola antibodies. Milk production data recorded at each herd testing event (n=3-4 per herd) were analysed for each cow.

5.2. 2 Herd Selection

Farms which were included in the cross-sectional study (Chapter 4; Herds A, B, H and I) and were known to have liver fluke infection in the cattle were considered to be suitable for the longitudinal study. The second requirement was that the farmer was willing to allow cows to be blood sampled in spring and autumn of the lactation and provide their herd testing data. Farmers were contacted by telephone and four herds were enrolled. Herds were assigned the same identifier as in the cross-sectional study.

5.2.3 Sample Collection

Cows were milked on a rotary platform with a continuous flow of cows entering and exiting with the PhD student standing on a fixed raised platform of 2 metre length on the outer circumference of the rotating platform. A total of 150 cows were sampled at the first visit, preferentially selecting those in their first and second lactation with the remainder being older cows, with all being identified by the numbered farm ear tag. For the autumn sampling, cows sampled in spring and still present in the lactating herd were identified during an afternoon milking with spray raddle applied to the rump before returning the following morning to sample those same cows.

Serum samples were transferred to an insulated carrier containing frozen pads to be sent to the laboratory on overnight courier on the day of sampling except the last farm sampled on each week which were transported by car to Massey University. On arrival at the laboratory the blood samples were centrifuged at 1,100 g for 15 minutes (Thermo Scientific, Heraeus Megafuge 40) and pipetted into labelled 1.5mL Eppendorf tubes. Paired samples of serum from each animal were stored at -20°C.

5.2.4 Cow Breed Categorisation

As described in Section 4.2.4

5.2.5 Herd testing data

As described in Section 4.2.5

5.2.6 Testing Procedure

The two test protocols used were the IDEXX antibody ELISA (IDEXX Fasciolosis Verification, IDEXX Europe BV, Hoofdorp, The Netherlands) and the In-House ELISA which were both undertaken as described in Section 4.2.7.1 and Section 4.2.7.3 respectively.

5.2.7 Calculating the economic impact of liver fluke infection on milk production

In the 2017/18 lactation the average herd supplying Westland Milk had 415 cows and produced 4,150 litres of milk per cow (https://www.westland.co.,nz/people-and-place/our-farmer-suppliers/overview). The values of $6.75/kg for milk solids, comprising milk protein at $7.6506/kg and milk fat at $6.044/kg were used in calculations of the economic cost of infection. These values were those to dairy farmers in New Zealand during 2022/23.

5.2.8 Statistical Analysis

A model of the lactational change in milk production parameters was developed, describing the lactation curve for each milk component using R (R Core Team, (2022). R: A Language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/). The data structure itself was hierarchical and had 3 levels of information and potential variability, the 1st level was the repeated herd tests on individual cows, the 2nd level was the individual cows and the 3rd level was the herds from which the individual cows were selected.

Models of each milk production parameter (ECM, milk solids (MS) milk fat % (MF%), milk protein % (MP%), milk lactose % (ML%)) were first created for all cows from all herds using a repeated measures random effects model in R using packages 'lme4' (Bates *et al.* 2014). The variable days in milk (DIM) was calculated as the number of days from the calving date to the date of each herd test. The variable was centred to reduce multicollinearity when two polynomial variables were created, DIM^2 and DIM^3. Lactation was used as a proxy for cow age with cows categorised by lactation as either first, second or third and more. Cow breed was categorised by the farmers description of each cow and herd test was categorised as 1,2,3 or 4. Since there were only 4 dairy herds herd was also entered as a fixed effect and each cow had a unique ID also entered as a variable. "CowHerd" was created by concatenating the cow ID and herd e.g., cow 68 from herd B became 68B. Herd was entered into the model a priori and the other fixed effects were tested and retained in the models if $p<0.05$, using the log likelihood ratio test (LRT), included DIM, lactation, breed and herd test. Cow was entered as a random effect and a random slope for DIM was tested and retained at $P<0.05$ and likelihood ratio test (LRT).

The following is an example of a model fitted to describe the lactation curve for Milk Solids (MS).

mod.lmer<-lmer(MS~DIM + Lactation + Herd +Test + Breed + DIM^2 + DIM^3+ (DIM|CowHerd), data=data)

Once a model was fitted which described the lactation curve of a milk component (MF, ML, MP, ECM), a variable describing the infection status of the cow, based on the results of the spring and autumn antibody testing, was then tested in the model. This variable describing liver fluke infection status based on the IDEXX diagnostic categories (Table 4.2) was created by first selecting all the cows that tested *negative* at the spring IDEXX ELISA test. Of these cows, those that were still in the *negative* category in autumn were categorised as 'uninfected' while those now in the *strong positive* category were categorised as 'infected'. The marginal effects of liver fluke infection on production were

estimated by comparing 'infected' to 'uninfected' categories using the 'emmeans' package across all four herds (Lenth, 2023) for each lactation parameter.

The model goodness of fit was determined by graphing the residuals and measuring the $R^{2.}$ Value.

The mean and quartiles of IDEXX SP% were calculated in Microsoft Excel (Microsoft Corporation (2018). Cohen's kappa coefficient (kappa) was used to measure inter-rater relatability of the IDEXX diagnostic categories of spring and autumn serum ELISA results from cows in the herds. Kappa was calculated in R, with interpretation of the values based on Landis and Koch (1977); < 0 as indicating *no agreement*, 0–0.20 as *slight agreement*, 0.21–0.40 as *fair agreement*, 0.41–0.60 as *moderate agreement*, 0.61–0.80 as *substantia agreement* and 0.81–1 as *almost perfect agreement*.

To assess the test characteristics of the In-House ELISA using the IDEXX ELISA as the gold standard, three datasets were compiled to be compared with each other. The first dataset ('2 herds Autumn') comprised cattle from Chapter 2 and Herd A from Chapter 3 (n=164). The second dataset ('Autumn') was a combination of samples from Chapter 4 sampled in 2018 (Herds A,B,C,D,E,F,G,I; n=909) and Chapter 5 sampled in 2019 (Herds A,B,D; n=385) collected in the autumn. The third dataset ('Spring') was a combination of samples from Chapter 5 (Herds A,B,D; n=385) collected in the spring. The positive cut-point (cutoff), sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV) and receiver operating characteristic (ROC) curve for each dataset were calculated in R using a *Yuoden* method and the packages "pROC' (Robin *et al.* 2011) and "OptimalCutpoints" (López-Ratón *et al.* 2014).

A two tailed t test was used to calculate the variance between the spring and autumn In-House ODR.

5.3 Results

5.3.1. Farm geography description

The characteristics of the geography of the farmland of the sampled herds are described in Table 5.1

Table 5.1 Description of the geography of the land the four herds in the study grazed.

Herd Code	Geography
A	Lakeside, prone to flooding
B	Well drained river flat
H	Poorly drained river flats, humped and hollowed
I	Valley floor, humped and hollowed

5.3.2 Cow sampling, lactation number and herd testing dates

Cows were blood sampled during November 2018 (spring) and March 2019 (autumn: Table 5.2) with a separation of 16 weeks, resulting in paired samples with milk production data from 485 cows across

the four herds. Cows were identified as being in their first (n=266), second (n=124) or subsequent (n=95) lactations.

Table 5.2 Dates herd tests were conducted with blood samples dates and the number of paired serum samples for each Herd.

Herd	Herd test dates				Blood sampling dates		Number of cows with paired samples
					Spring	Autumn	
A	30/09/2018	11/12/2018	12/02/2019	19/04/2019	12/11/2018	6/03/2019	126
B		19/12/2018	3/03/2019	2/05/2019	13/11/2018	8/03/2019	104
H	27/09/2018	5/12/2018	4/02/2019		14/11/2018	9/03/2019	129
I	24/09/2018	22/11/2018	20/01/2019	16/04/2019	15/11/2018	10/03/2019	155

Herd testing was conducted in all four herds (Table 5.3) with four tests on two farms and three tests in the remainder. A total of 1694 complete cow data points (herd test data and serum analysis IDEXX ELISA) from 485 cows were analysed. The number of cows per farm ranged from 102 – 153, representing 17-26% of the cows in each herd. For Herds B and H, three herd tests were carried out due to storms causing damage on the properties preventing testing, while in Herds A and I four herd tests were conducted.

Table 5.3 Cows present at each herd test with the number and percentage of cows present at any test.

Number of sampled cows present at each herd test				
Herd	Test 1	Test 2	Test 3	Test 4
A	122	126	126	116
B	---	103	104	102
H	92	101	101	---
I	143	155	154	149
Total	357	485	485	367

5.3.2 Average days in milk at the spring and autumn sample collection

Using calving dates provided by the farmers, the average (and range) days in milk (DIM) at the spring sampling was 83 (37-160), and for the autumn sampling 197 (151-274: Figure 5.1).

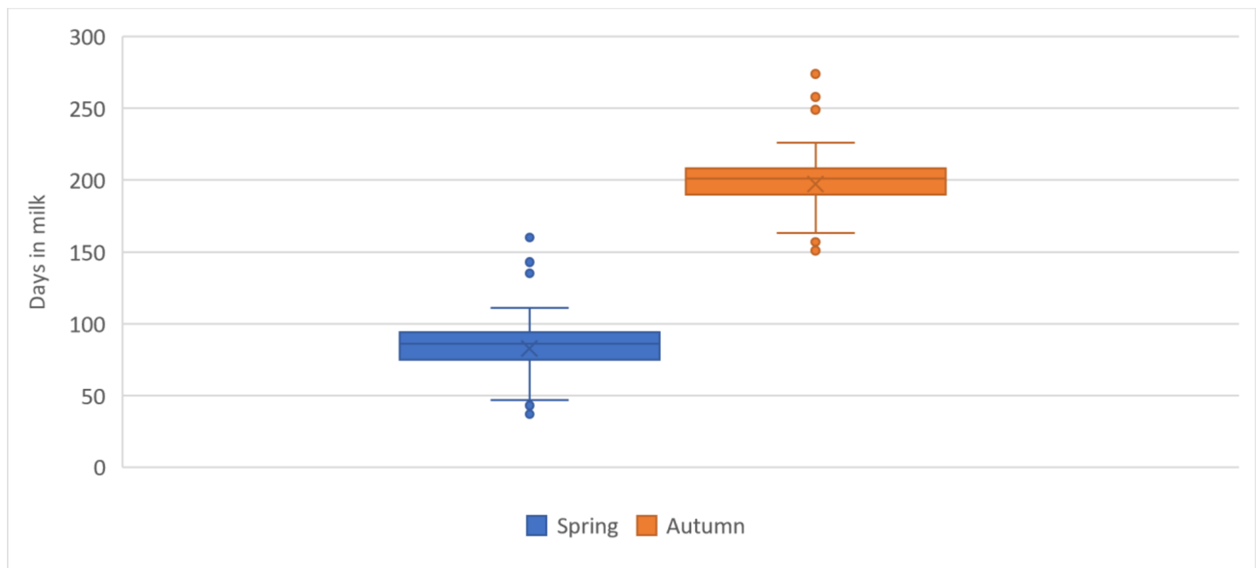

Figure 5.1 Box plots of days in milk (and mean) of all cows at the spring and autumn sample collection.

5.3.3 IDEXX antibody ELISA results

5.3.3.1 Change of IDEXX SP% from spring to autumn

Overall, there was an increase of the mean IDEXX SP% from spring to autumn of 67 points from 121 to 188 ($p<0.05$), with the change for individual cows between the two sampling points ranging from -625 to +565. The median SP% for each herd was greater in the autumn than spring (Figure 5.2).

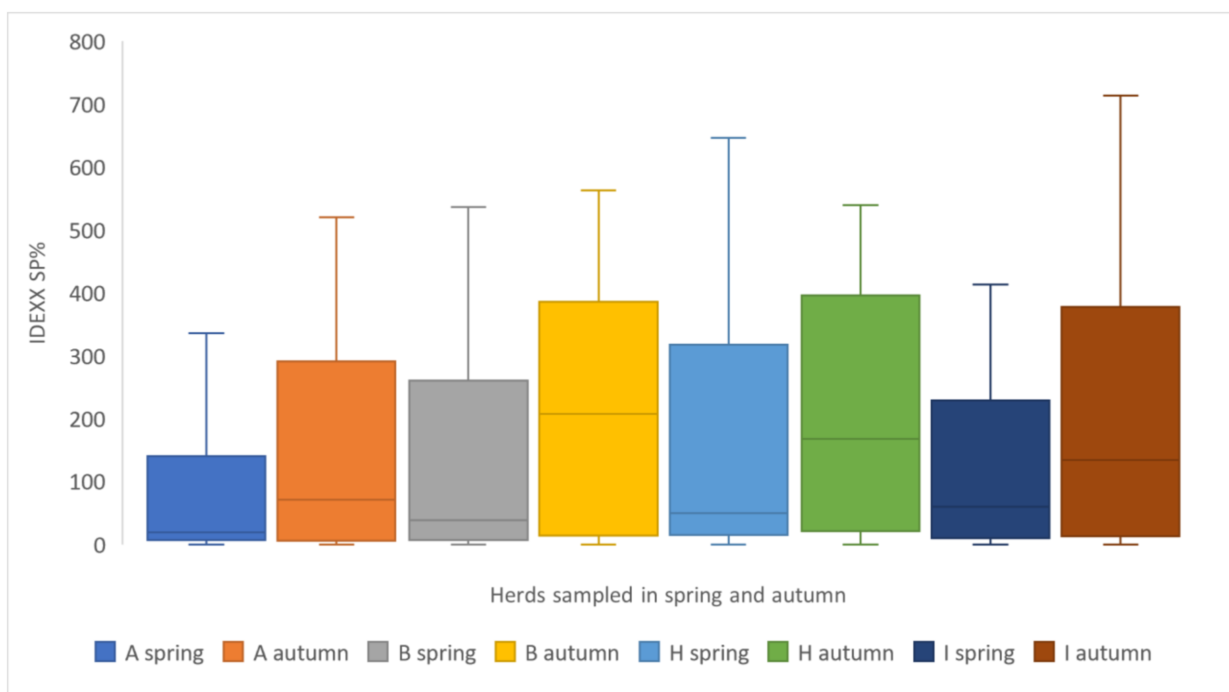

Figure 5.2 Box plots of IDEXX SP% from cows sampled in both spring and autumn for the four herds.

The mean increase of IDEXX SP% between the two sampling points for Herd A was 83 (95%CI 69-98), for Herd B was 32 (95%CI 10-54), for Herd H was 13 (95%CI -10-36), and for Herd I was 38 (95%CI 19-56). The mean increase of SP% in Herds B and I was significantly less than for Herds A and H (p=0.005). The adjusted R^2 of 0.29 indicates there is considerable variation between the IDEXX SP% change between the four herds.

5.3.3.2 Change of IDEXX diagnostic category from spring to autumn

There was a decrease in the percentage of cows in the *negative*, *mild positive* and *positive* IDEXX categories with an increase of the *strong positive* category between spring and autumn. The percentage of cows in each diagnostic category are skewed toward either the *negative* or *strong positive* diagnostic categories with only 18% and 14% of cows in the *mild positive* and *positive* diagnostic categories at the spring and autumn sampling, respectively (Table 5.4).

Table 5.4 The number of cows in each IDEXX diagnostic category both autumn and spring. For the diagnostic categories, the figures in parenthesis indicate the percentage of cows from the spring category in each category in autumn (calculated across the row). The total figures are for all results for spring and autumn with the figures in parenthesis indicating the percentage in each diagnostic category for spring and autumn. Percentages may not equal 100 due to rounding.

		Autumn IDEXX Category				
		Negative	**Mild positive**	**Positive**	**Strong Positive**	**Total**
Spring IDEXX Category	**Negative**	152 (65%)	24 (10%)	9 (4%)	51 (21%)	235 (48%)
	Mild positive	13 (30%)	5 (12%)	5 (12%)	20 (47%)	43 (9%)
	Positive	5 (11%)	1 (2%)	8 (18%)	30 (68%)	44 (9%)
	Strong positive	14 (9%)	8 (5%)	8 (5%)	133 (82%)	163 (34%)
	Total	184 (38%)	38 (8%)	30 (6%)	233 (48%)	485

Between the spring and autumn 62% of cows had the same IDEXX diagnostic category, 13% decreased one to three categories and 28% increased one to three categories (Table 5.5).

Table 5.5 The percentage of cows that either decreased, increased or remained in the IDEXX diagnostic category between the two sampling points. If a cow decreased three categories, this is depicted as -3.

IDEXX category variance	-3	-2	-1	0	+1	+2	+3
Percentage of total cows	3%	3%	4%	62%	12%	6%	10%

5.3.3.3 Cohen's kappa analysis of agreement of IDEXX diagnostic category for all herds

Over all herds there was a *moderate agreement* between the IDEXX diagnostic category of the spring and autumn sampling points (Table 5.7) with a weighted kappa = 0.51 (95% CI, 0.46 to 0.57; p <

0.0001). For the individual herds there was a *moderate agreement* for Herds A, B and I and a *fair agreement* for Herd H between the IDEXX diagnostic category of the spring and autumn sampling points (Table 5.6).

Table 5.6 Weighted kappa of the IDEXX diagnostic category of each herd in spring and autumn and for combined herds including lower and upper 95% confidence intervals (CI).

Herd	Weighted kappa	Agreement strength	Lower 95% CI	Upper 95% CI	P value
A	0.48	Moderate	0.36	0.60	p<0.0001
B	0.55	Moderate	0.42	0.69	p<0.0001
H	0.37	Fair	0.24	0.50	p<0.0001
I	0.57	Moderate	0.47	0.68	p<0.0001
All herds	0.51	Moderate	0.45	0.57	p<0.0001

5.3.3.4 IDEXX SP%, diagnostic category and cow lactation

First lactation cows had higher SP% at both sample points ($p<0.05$), the mean SP% used to determine the diagnostic category of the herd for all ages were in the *positive* diagnostic category in the spring sampling and increased to *strong positive* by the autumn sampling (Table 5.7).

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Table 5.7 Number and percentage of cows in their first, second, or subsequent lactation, with the mean Spring and Autumn IDEXX SP% and resulting diagnostic category for that value for each.

		Number of cows in each age category (and percentage of total cows)	Spring		Autumn	
			Mean IDEXX SP%	IDEXX category	Mean IDEXX SP%	IDEXX category
Lactation	First	266 (55%)	142	*positive*	196	*strong positive*
	Second	124 (26%)	95	*positive*	179	*strong positive*
	Third and more	95 (20%)	94	*positive*	177	*strong positive*
	Total	485	121	positive	188	strong positive

5.3.4 In-House ELISA results

In total, 480 cows had paired In-House antibody ELISA results (Table 5.8). At the spring sampling 31% were positive (ODR ≥0.42) with 35% positive in autumn.

Table 5.8 Number (and percentage) of the 486 cows in each In-House ELISA diagnostic category in spring and autumn with a positive cut point ODR ≥0.42.

In-House ELISA ODR (and % in each category)		
	Negative	Positive
Spring	333 (69%)	147 (31%)
Autumn	313 (65%)	167 (35%)

Overall, there was an increase of the mean ODR from spring to autumn of 0.09 points from 0.33 to 0.42 (p=0.003), with the change for individual cows between the two sampling points ranging from -1.54 to 2.68. The median ODR was greater in the autumn than spring for Herds A and B but lower for Herds H and I (Figure 5.3).

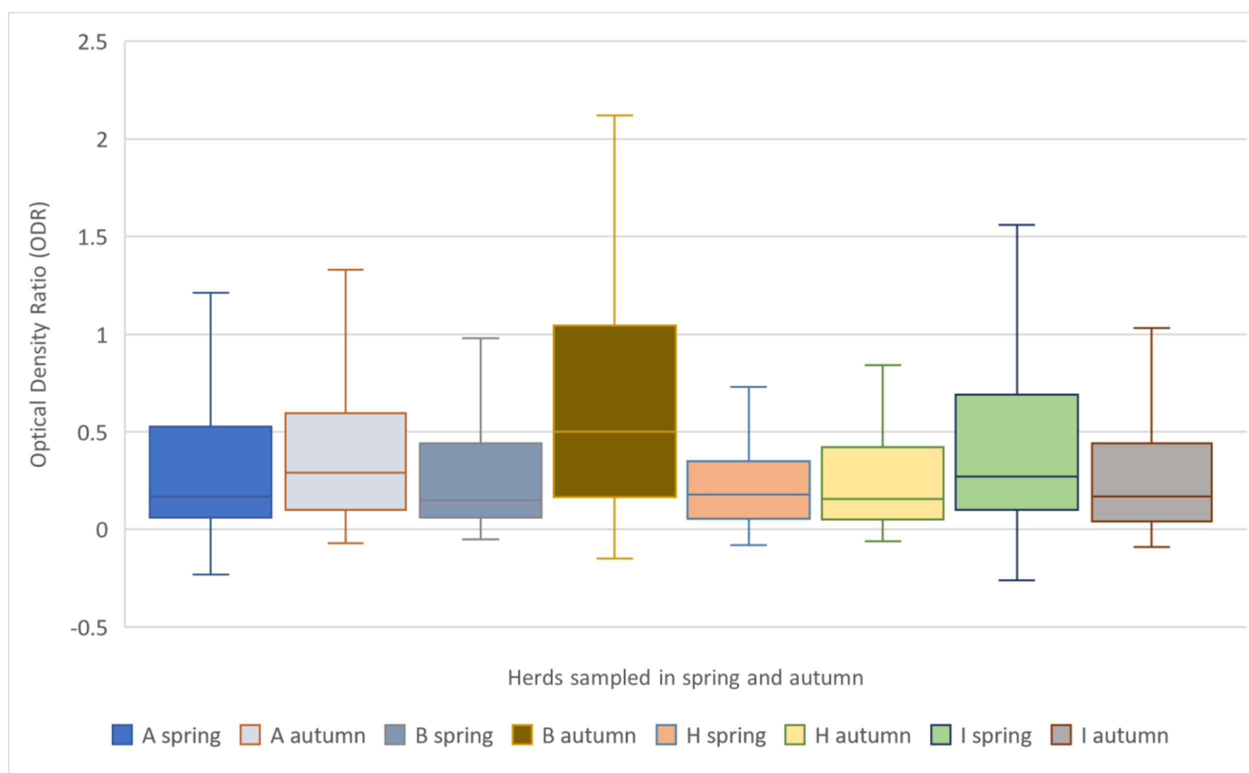

Figure 5.3 Box plots of In-House ELISA ODR from cows sampled in both spring and autumn for the four herds.

5.3.5 Lactation Curves

Milk solids (MS) production per cow per appears similar to that described by Silvestre (2009) without the early season "peak" (Figure 5.4).

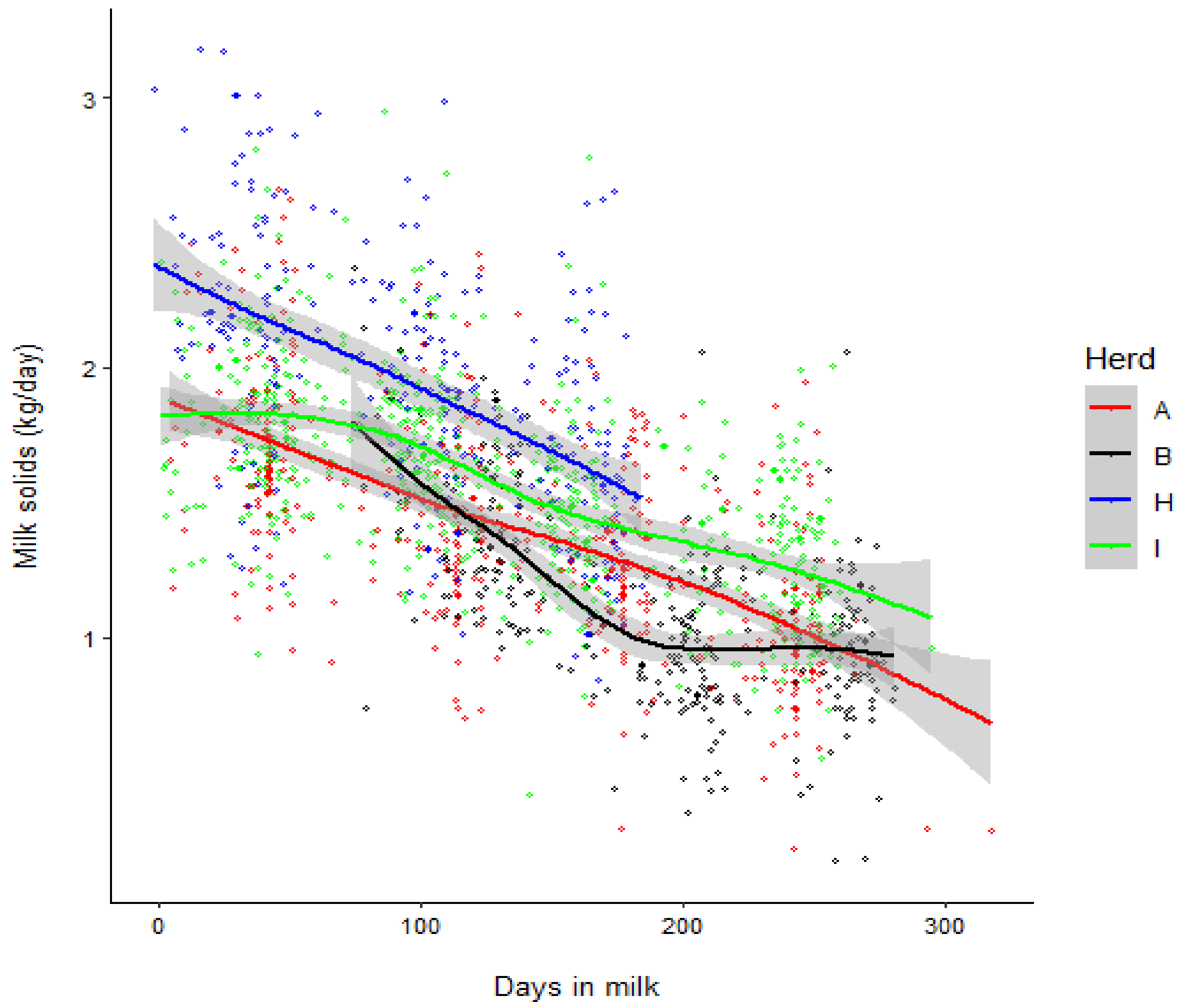

Figure 5.4 Milk Solids (kg/cow/day) production from each cow herd test over the length of the lactation with the mean result indicated by trend lines for each herd A, B, H and I.

Milk protein percentage (MP%; Figure 5.5) appear similar to the “Nike tick” pattern of a normal lactation curve as described by Silvestre (2009).

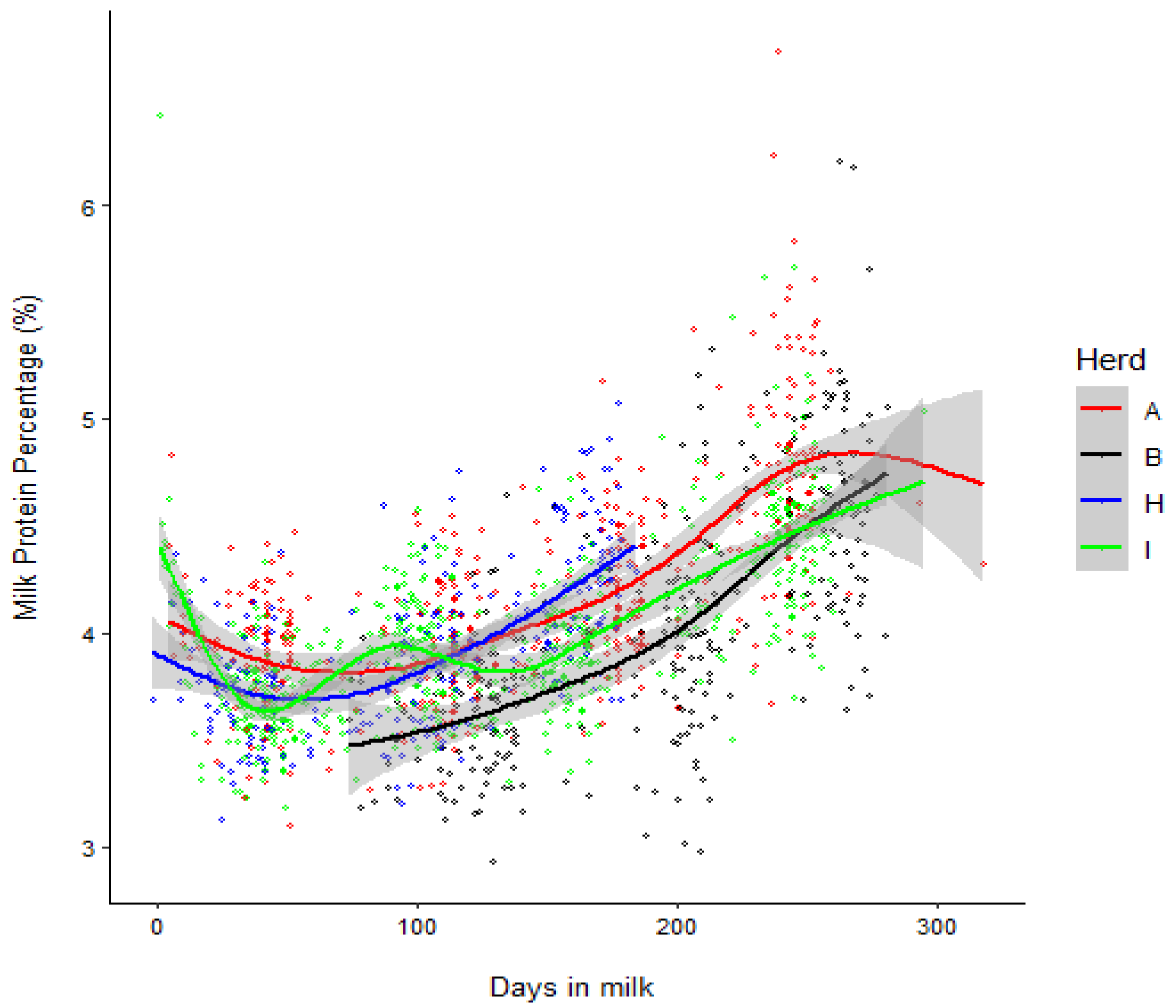

Figure 5.5 Milk protein percentage (%/cow/day) from each cow herd test over the length of the lactation with the mean result indicated by trend lines for each herd A, B, H and I.

The pattern of milk fat percentage (MF% Figure 5.6) is similar to that described by Silvestre (2009).

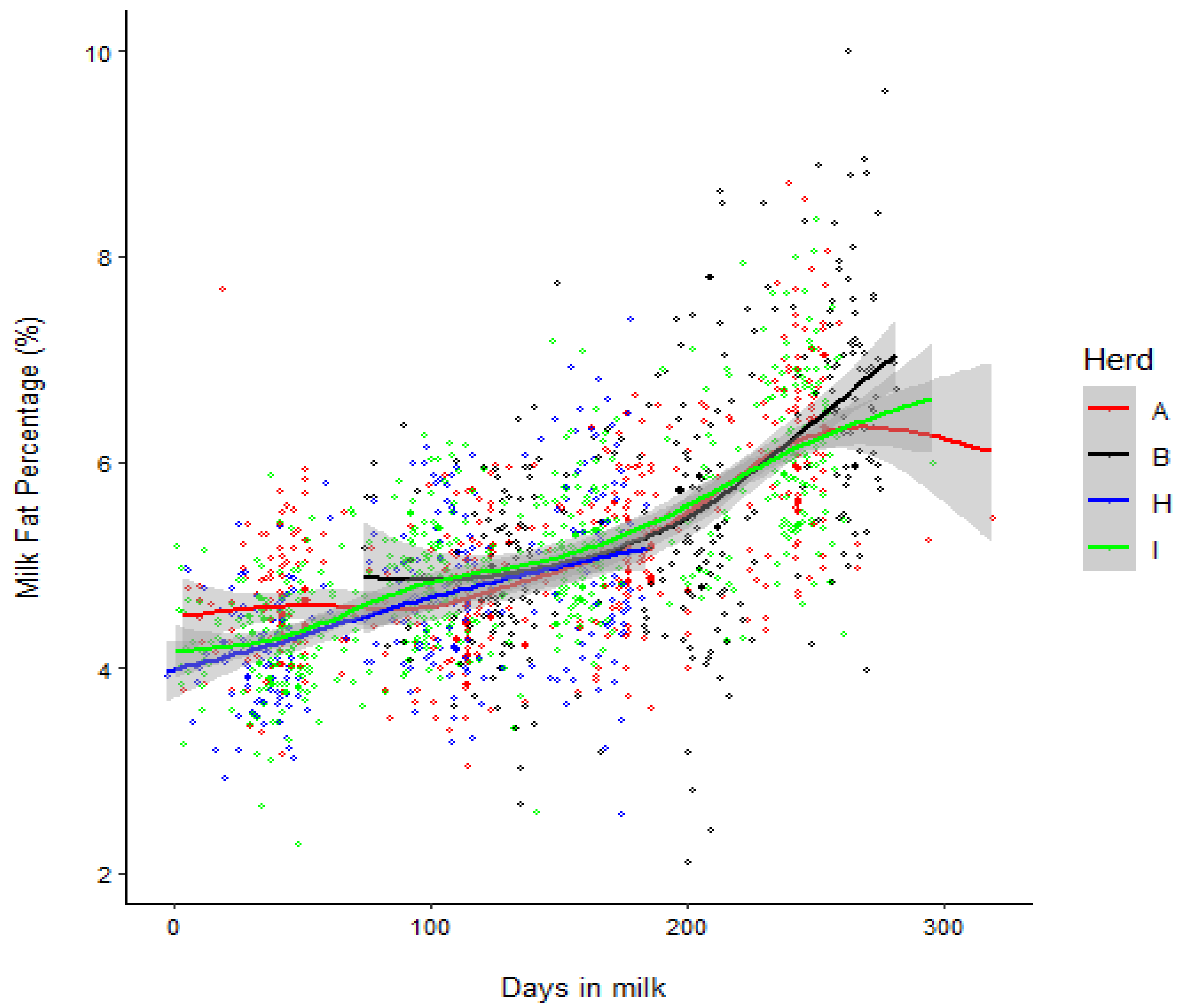

Figure 5.6 Milk fat percentage (%/cow/day) from each cow herd test over the length of the lactation with the mean result indicated by trend lines for each herd A, B, H and I.

Milk lactose percentage (ML%) rose and declined over the lactation (Figure 5.7).

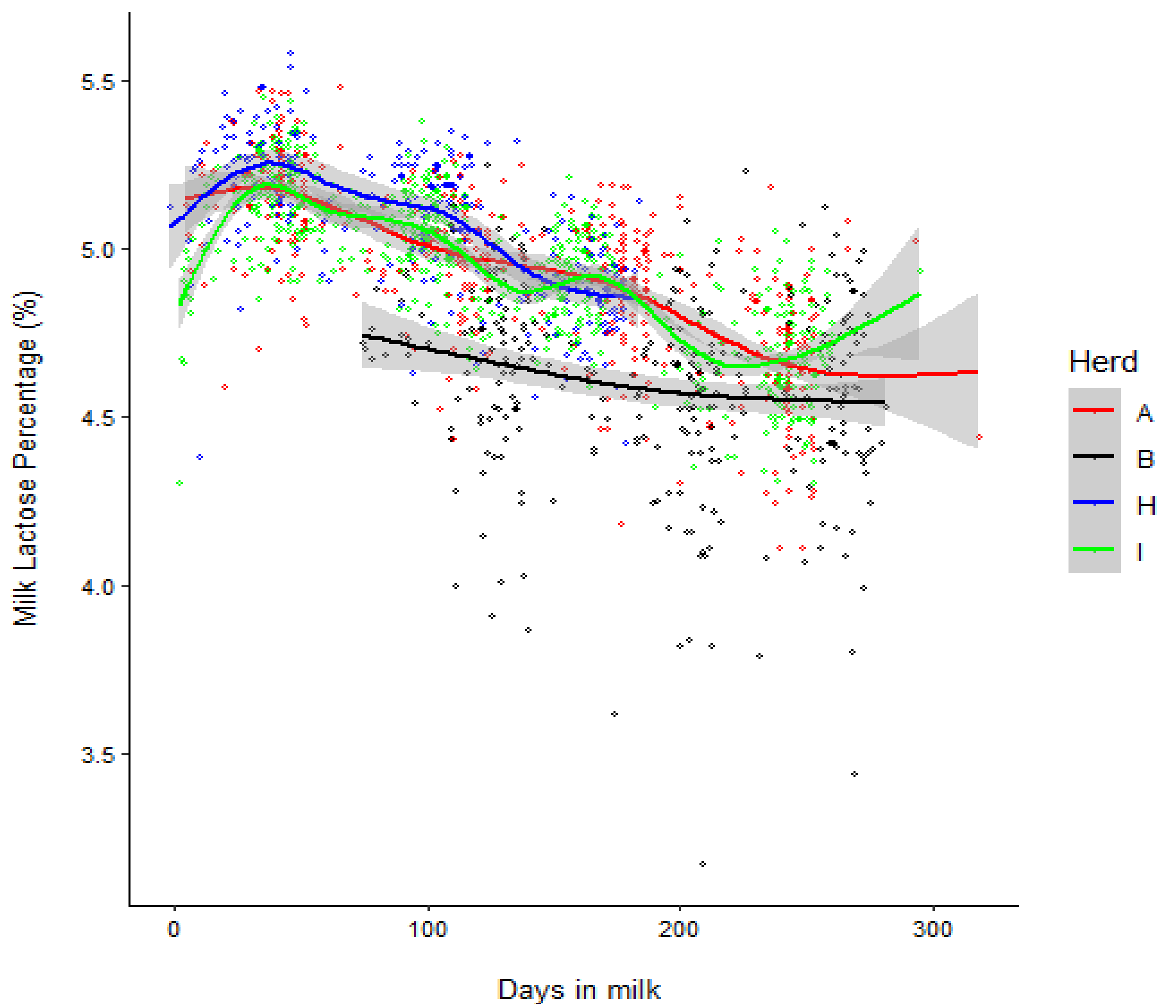

Figure 5.7 Milk lactose percentage (%/cow/day) from each cow herd test over the length of the lactation with the mean result indicated by trend lines for each herd A, B, H and I.

5.3.6 IDEXX antibody ELISA and milk testing data

A total of 235 cows were categorised as uninfected at the spring testing, being in the *negative* diagnostic category. Of those 235, at the autumn testing 152 remained in the *negative* diagnostic category and 51 were in the strong *positive diagnostic* category. This gives 152 cows that were *negative*/*negative* and classified as 'uninfected' and 51 cows that were *negative*/*strong positive* and were categorised as 'infected'. The different models showed no effect of change of infection status on ECM (p=0.40), on MS (p=0.28), ML% (p=0.20), or on MP% (p=0.07). However, there was support for a significant effect of change of infection status on MF% (p=0.014) with the mean MF% being 0.22 MF% points (95% CI 0.05 – 0.53%) lower for 'infected' compared to 'uninfected' cows.

5.3.7 Calculating the economic cost of liver fluke infection on milk production

A 0.22% point reduction in milk fat in an 'infected" cow equates to 2.2g MF/L lower production compared to an 'uninfected' cow. For the average herd (Section 5.2.7) this represents a loss of 3,789.5 kgMF (1,722,500 litres * 2.2/1000 = 3,789.5kgMF) with a value of $6.044/kgMF this represents an economic loss of $22,903.74 where all cows are infected, or $55.19 per cow in an average herd of 415 cows (Table 5.9).

Table 5.9 The economic cost of liver fluke infection on milk fat calculated at the level of the herd (mean n=415 cows) and cow level for varying prevalence of infection within the herd.

Prevalence of infection within the herd	Impact on loss of milk fat at a herd level	Impact on loss of milk fat at a cow level for all 415 cows
0%	0	
20%	$4,580.75	$11.03
50%	$11451.87	$27.60
100%	$22,903.74	$55.19

5.3.7 In-House ELISA test characteristics

The test characteristics including optimal positive cut-point, Se, Sp, PPV, NPV and area under the curve calculated by ROC analysis for all three datasets are presented in Table 5.10.

Table 5.10 Calculated positive cut-point (Cutoff), sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV), area under the curve (AUC)of the In-House ELISA using the IDEXX ELISA as a gold standard for the three datasets; '2 herds Autumn', 'Autumn' and 'Spring'.

	2 herds Autumn	Autumn	Spring
Positive cut-point	0.42	0.3	0.23
Se	0.81 (0.74-0.87)	0.74 (0.71-0.77)	0.70 (0.64-0.76)
Sp	0.9 (0.80-0.95)	0.79 (0.76-0.83)	0.70 (0.63-0.76)
PPV	0.95 (0.89-0.97)	0.85 (0.83-0.87)	0.70 (0.63-0.76)
NPV	0.67 (0.57-0.85)	0.66(0.62-0.70)	0.70 (0.63-0.77)
AUC	0.90 (0.85-0.94)	0.84 (0.82-0.86)	0.76 (0.72-0.81)

The ROC curves B ('Autumn') and C ('Spring') indicate that the In-House ELISA is a poor predictor of the IDEXX ELISA (Figure 5.8). Of the three curves, curve A ('2 herds Autumn') looked most promising to give good values but was compromised as one of the two herds contained all positive results.

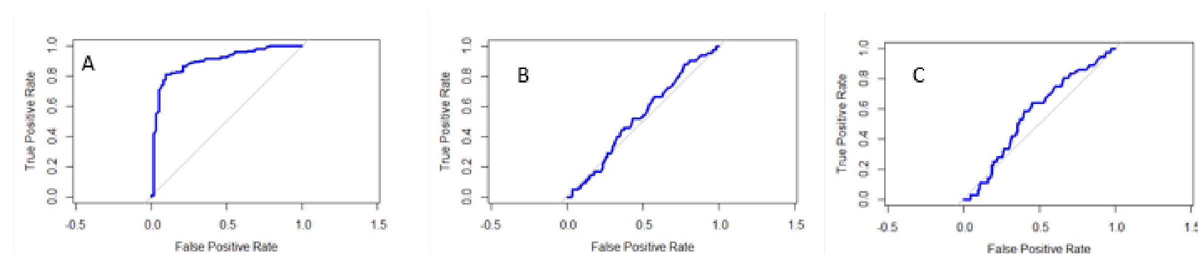

Figure 5.8 Receiver operating characteristic (ROC) curves for the three datasets in Table 5.9 Curve A is calculated from '2 herds Autumn", curve B 'Autumn' and Curve C 'Spring"'. The closer the calculated blue line is to the grey random classifier line, the less predictive the In-House ELISA is of the IDEXX ELISA.

5.4 Discussion

There was relatively minor change of IDEXX diagnostic category for all cows over the four months between sampling points, with 48% *negative* and 34*% strong positive* in spring, while in the autumn 38% were *negative* and 48% *strong positive*. A significant negative association was found between MF% and cows that were 'negative' in spring and 'positive' in autumn compared to those that were 'negative' for both spring and autumn, with a calculated economic cost of $55.19 per 'infected' cow per lactation. Although the younger 'first lactation' cattle had higher SP% at both sampling points than 'second lactation' and 'third and more lactation' cows, the interpretation was the same diagnostic category across all ages, being *positive* in spring and *strong positive* in autumn. Analysis of the In-House ELISA test found it to be a poor predictor of the IDEXX ELISA test.

5.4.1 Change of IDEXX antibody concentration between spring and autumn

The mean IDEXX ELISA SP% of all cows increased by 67, from 121 in the spring which is categorised as a *positive* result, to 188 in the autumn, which is categorised as a *strong positive* result indicating an increase in antibody titre over the lactation. In addition, there was a *moderate* agreement of the diagnostic categories between the two sampling occasions for Herds A, B and I while for Herd H there was a *fair* agreement (Table 5.7) which indicates that overall, the intensity of antibody response was not markedly different between the sampling points. Taken together, these two results suggest there may have been some exposure to further infection over lactation which stimulated a greater antibody response. However, it may also represent a maturing antibody response from an earlier infection. Similar results where the prevalence of infection has not changed markedly between seasons has also been reported in Ireland (Byrne *et al.* 2018), Belgium (Charlier *et al.* 2008) and Germany (Kuerpick *et al.* 2012b), where in each there was an increase over the course of the study as also seen in the current study.

Seasonal patterns of host response to liver fluke infection using bulk milk antibody ELISA have been reported with two Irish studies having the highest antibody levels detected in winter compared to the other seasons (Byrne *et al.* 2018; Munita 2019) while in northern Germany the autumn levels were higher in one study (Kuerpick *et al.* 2012a). Similarly there was a marked seasonal variation in fluke prevalence found in Portugal, being highest in spring to summer (Conceicao *et al.* 2004) and in Austria in spring (Kostenberger *et al.* 2017) . However, the farming system in these other locations is different to that in New Zealand as cattle are generally housed over winter in Europe, preventing reinfection with metacercariae and allowing the current fluke burden to mature if not effectively treated. This suggests that when housed cattle have a higher antibody titre in winter, this may be caused by a maturing of the infection present at the commencement of housing. Others have also proposed that an increase in the antibody concentration in the host does not always mean that cattle are becoming infected with more fluke, it may be that those infected have a greater immune response (Byrne *et al.* 2018) with this being related more to host factors than the current fluke burden. The persistence of the antibody response in cattle confuses the interpretation of serological responses and how it co-ordinates with cattle either maintaining an infection or becoming either reinfected or further infected. Liver fluke infection is dependent on development and persistence of metacercariae which is generally weather dependent, and ill-defined in New Zealand. It was notable that for the year of the current study, the spring period was unusually dry for the West Coast (Rainfall Stats NZ). The Hokitika weather monitoring station, being the closest to Herds A, B and H in this study, shows a considerable variation

of annual rainfall of between 2300 and 3600 mm/year. However, spring and summer rainfall of 2018/19 were 198 and 274mm less, whilst autumn was 369mm more, than the 30-year mean (https://www.stats.govt.nz/indicators/rainfall). This prolonged dry period over spring and summer for those herds likely resulted in a lower mud snail population resulting in fewer infective metacercariae on pasture over the summer period. This would account in part for the less than expected change of infection status and SP% between the two sampling points, reflected in 62% of cows remaining in the same diagnostic category (Table 5.6). The wetter autumn was likely to have resulted in greater infection intensity in cows later in the autumn and was noted in BME results on some farms in the early winter (data not shown). The growing degree days monitored from the Hokitika station were also above the 30-year mean (Growing degree days | Stats NZ), favouring the proliferation of snails if moisture is sufficient. Although there have been no studies of snail ecology/epidemiology on the West Coast it is presumed that temperatures would be warm enough over lactation for snails to survive, reproduce and harbour developing *F. hepatica.* Overseas studies have shown the importance of rainfall for fluke epidemiology. In Zambia the percentage of cattle that had flukes detected in the liver was three times greater after the wet season as compared to after the dry season (Phiri *et al.* 2005) with the wet season favouring proliferation of the intermediate host. The relationship of fluke infection and conditions suiting the intermediate host snail were also postulated as the reason why fluke transmission in Louisiana, USA occurred from February to July (Malone *et al.* 1984b) .

Interestingly, 10% of cows decreased at least one diagnostic category without any treatment, either as a result of self-cure or natural variations of the circulating antibody concentration on the days of sampling. As indicated earlier, the 28% that increased one or more diagnostic categories (Table 5.6) may be due to new infection or just further development of the hosts immune response to the same number of flukes as has been previously reported for the IDEXX ELISA (Munita 2019). This study was not designed to be able to assess which is the more likely in either group of these cows.

The relative lack of change of anti-fasciola antibodies between spring and autumn makes detection of milk production effects associated with a change of antibody titre less likely and poses a limitation on the original aim of this study. In studies where cows are housed for extended periods it is more likely that the anti-fasciola antibody titres would decrease due to antibody decay and an absence of metacercarial ingestion, to be lower at the end of the housing period, and then increase once cows resume to grazing infected pasture.

As was also noted in Chapter 4, the IDEXX antibody ELISA categorised cows largely into either the *negative* or *strong positive* categories, with relatively few in the *mild positive* and *positive*. Again, this indicates that the IDEXX antibody ELISA test may be over-interpreting the antibody titre to assign an animal to a diagnostic category and that just the categories negative and positive may be more suitable. In addition, results in Chapter 2 found no association between serum IDEXX SP% and total fluke count in 39 cattle.

5.4.2 Higher IDEXX SP% in first lactation cattle

The higher mean spring IDEXX SP% in cattle in their first lactation relative to the two older groups of cattle (Table 5.8) could be the result of this age group having a greater number of flukes present, but equally may be due to naïve animals being exposed to the parasite. The difference was largely absent

in autumn. As has been discussed in this thesis, the antibody ELISA is not a quantitative diagnostic tool so any difference between the age groups, if there is any at all, requires further investigation using the coproantigen ELISA.

5.4.3 Impacts on milk production.

Unfortunately, two of the four herds performed only three of the expected four herd tests in the lactation due to adverse weather events preventing testing, weakening the dataset.

Using a categorical variable to capture the change from 'uninfected' to 'infected' (section 5.3.6), from spring to autumn, over the lactation showed a negative effect on MF% with infected cows being 0.22 MF% points lower. This is consistent with the relationship between IDEXX SP% and MF% shown in Chapter 4 although is a higher value than that seen in the earlier chapter (0.05 MF% points difference between high and low cut points). Milk fat % was also seen to decrease in a Belgian study where bulk milk ELISA was used to determine anti-fasciola antibody concentration in the spring in 463 herds (Charlier *et al.* 2007). In that study, herds in the 75th percentile of results had a decreased MF% of 0.06% compared to cows in the 25th percentile. Similarly, an Austrian study assessing milk production parameters against a bulk milk ELISA analysis in 178 herds in the spring demonstrated a 2.2% lower MF% in herds that were considered to have a high positive result (Kostenberger *et al.* 2017). Milk fat is a component of the milk solids calculation from which New Zealand farmers are paid. Thus, an effect on milk fat will have a negative effect on milk value to the farmer, with a calculated cost of $55.19 per cow per lactation. For the average herd supplying Westland Milk Co-Op with 415 cows, if all cows were infected this multiplies to $22,903.74. However, this only reflects the presence of anti-fasciola antibodies in the current lactation so may underestimate the true impact of infection as cows may already have hepatic insufficiency due to previous liver fluke infections. Farmers and their veterinarians can use the results of a BME to estimate the prevalence of infection within a herd to determine if there is an economic benefit to investing in methods to reduce the likelihood of cows becoming infected.

5.4.4 Assessment of the In-House ELISA diagnostic test

These two ELISA assays detect antibodies specific to excretory secretory antigens (ESA) produced by the liver flukes. The IDEXX ELISA utilises the f2 fraction of the ESA and has both high sensitivity (82-99%) and specificity (80-99.3%; Table 1.13). The In-House ELISA uses unrefined ESA collected from flukes as described by Salimi-Bejestani (2005b), having a near perfect agreement with the commercial Bio-X bovine *F. hepatica* ELISA kit (BioK 211 kit, Bio-X Diagnostics, S.A rue de la Calestienne, 38 (PAE) 5580 Rochefort), a monoclonal antibody ELISA with an affinity for fraction IV of the CL1 component of the ESA. However, as reported in Chapter 2, the In-House ELISA showed a low sensitivity when compared to the IDEXX ELISA. For this reason, the IDEXX ELISA became the test of choice for the remaining studies and was used to interpret the relationship between serological values and milk production characteristics. This In-House methodology, or variants of it, referred to as the LSTM ELISA (it was developed at the Liverpool School of Tropical Medicine) is the most commonly used antibody ELISA test in published studies (Sekiya 2013). In the present study this assay showed a similar overall increase in OD values over the lactation to that shown by the IDEXX ELISA.

The test characteristics of two of the datasets 'Autumn' and 'Spring' are poor. However, the results of dataset A ('2 herds Autumn') looked more promising but the absence of any negative results in one of the herds means these results should be discarded. The reasons for the poor calculated characteristics of the In-House ELISA (Table 5.15: Figure 5.6) include two possible reasons. The first is poor performance of the In-House assay itself and the second is the use of an imperfect reference (='gold') standard test to which it is compared. To the first point, the In-House assay uses unrefined excretory-secretory antigens (ESA) which may also contain other non-fasciola proteins whereas the IDEXX ELISA uses a refined f2 fraction, which was found to be superior to other diagnostics using unrefined ESA (Levieux *et al.* 1992a). To the second point, the IDEXX ELISA was also shown to be an imperfect test in Chapter 2, and the use of such a test as a 'gold' standard to determine the test characteristics of a new test will tend to overestimate the Se and Sp to reflect itself (Rapsch *et al.* 2006; Mazeri *et al.* 2016).

Variance in test characteristics related to season of testing as seen in the 'autumn' and 'spring' datasets of this current study has been noted before (Charlier *et al.* 2012) and warrants further investigation.

These results all lead to the conclusion that the In-House ELISA is a poor diagnostic tool as was previously indicated in Chapter 2.

5.4.5 Summary

The increase of IDEXX SP% between the spring and autumn was of a lower magnitude than expected, probably due to the low rainfall over the period. There was an effect on MF% for cows which converted from a 'negative' category to a 'positive' status over that lactation with an associated loss of income of $55.10 per infected cow.

5.5 Animal Ethics

This study was performed under the approval of Massey University Animal Ethics Committee, Protocol MUAEC 18/36.

5.6 Supplementary Materials

Supplementary materials for Chapter 5 are contained in Section 3.2.5.

Chapter 6: The use of Bayesian latent class models to estimate the sensitivity and specificity of four liver fluke diagnostic tests under New Zealand field conditions.

6.1 Introduction

The liver fluke *Fasciola hepatica* is a trematode parasite of farmed livestock with worldwide distribution causing chronic production losses and possible death as a result of hepatobiliary damage (Charlier *et al.* 2014). The lifecycle of *Fasciola hepatica* is well described (Charleston 1997) with the adult fluke resident in the bile ducts of the infected animal and the intermediate host mud snails *P. columella* and *L. tomentosa* found in permanently wet areas. The intensity and geographical spread of liver fluke infection of cattle in New Zealand has been surveyed once using slaughter house recording (Charleston *et al.* 1990) and is predicted to increase due to climate change (Haydock 2016) with previously naïve areas being more suitable to the survival and flourishing of the intermediate host snails. This will result in some regions experiencing liver fluke infection for the first time, becoming another production limiting factor in their farm system. Effective management of liver fluke infection requires diagnostic tests which can accurately estimate the current infection intensity at both the individual and herd level, aspects complicated by factors including a two-host lifecycle, migratory phase in the host, long prepatent period and variable host immune response to the presence of the parasite.

There is an excellent test to determine *F. hepatica* infection on post-mortem material, the total fluke count (TFC), where the liver is sliced into thin sections and massaged to remove flukes, with a recorded sensitivity (Se) of 99% and specificity (Sp) of 98% (Mazeri *et al.* 2016). However, the test is only useful for research purposes, being too impractical for use as a diagnostic tool in the field due to the time taken, expense to examine each liver and the requirement for post-mortem material. The resulting diagnosis from TFCs is highly indicative of the state of infection in that animal but, as has been shown in Chapter 2, the range of infection within a cohort of animals can vary greatly from those with no flukes present to those with many. Therefore, the TFC is most suited to being used as a gold standard against which the test characteristics of a new diagnostic test can be measured.

The requirement for high test characteristics of Se and Sp is dependent on the purpose of the test and the disease prevalence. In a situation where disease is to be eradicated, a high Sp is required to ensure that very few positive animals are missed, keeping in mind that several uninfected animals may be treated or culled unnecessarily which also has a cost. When disease prevalence is high the Sp of the diagnostic test can have a greater impact on test outcomes compared to populations where disease prevalence is lower (Bentley *et al.* 2012). Often though, the prevalence of infection in the population is unknown. Diagnostic assays are also invariably developed and tested using artificial infections in a naïve population and then compared to an uninfected population. They are often compared to a non-gold standard test which can over- or underestimate the Se and Sp of the new test. As discussed in Section 1.1.6 (Host antibody response to Excretory Secretory Antigens (ESA)), the host response to an artificial infection can be quite different to that of naturally infected animals, adding to the complexity, as many manufacturers publish the results for their diagnostic test based on trials involving artificial infections. This indicates that tests must also be evaluated in the field, on animals that are naturally infected with multiple stages of the parasite life cycle.

There currently is no gold standard test for diagnosing liver fluke infection in live animals in the field (Mazeri *et al.* 2016). Antibody detection ELISA in either milk or serum, and faecal egg counting (FEC) are two commonly used diagnostic tools for determining the presence and intensity of infection in farmed livestock, with neither of these tests being sufficiently robust to be considered a gold standard. Furthermore, the test characteristics for these two tests has never been assessed on natural infections in New Zealand.

When undertaking a FEC, it is important to consider the dynamics of egg excretion. Egg output from flukes in cattle peak 18-20 weeks post infection (wpi) before decreasing to very low levels at 38-40 wpi (Bouvry and Rau 1986; Boulard *et al.* 1995). This means that false negative results, i.e., no eggs observed but adult flukes still present in the bile ducts, are more common in long-standing chronic infections or when the fluke burden is low (Kelly *et al.* 2021). Antibody ELISA assays detect the presence of circulating antibodies produced by the host in response to the presence of the flukes (Walsh *et al.* 2021). These antibody ELISAs have inherent problems which can manifest as false negatives due to delays in the activation of the immune system and production of detectable antibody titres and false positives as the antibodies will persist for a period after active infection has been eliminated, either after successful treatment or self-cure. The length of the delay depends on the magnitude of the antibody response which is greatly influenced by factors including: naivety of the host; number of metacercariae ingested and whether this is as an experimental bolus, trickle, or a natural infection (Walsh *et al.* 2021); stages of the fluke lifecycle present (Robinson *et al.* 2008; Kuerpick *et al.* 2013a; Cwiklinski *et al.* 2015; Walsh *et al.* 2021); immune capacity of the host; and the season of sampling (Charlier *et al.* 2008). The decay of antibodies cannot be accurately determined in part due to the unknown titre at the time of elimination of the flukes, host variability and detection sensitivity of the test used. This makes the antibody ELISA assays an indicator of both recent and current infections but cannot distinguish between the two and a poor choice for a gold standard test.

A more recent liver fluke diagnostic development is an antigen ELISA assay which detects the presence of excretory - secretory antigens (ESA) in either serum or faeces of infected people and animals. The ESA requires the presence of flukes or parts of fluke bodies in the hepatic parenchyma (for detection in serum) and biliary system (for detection in faeces) and a positive result indicates current or very recent infection where not all parts of fluke bodies have been eliminated from the host. Since this test does not rely on the host's immune response but infers infection by observing antigen, it is not plagued by the delay/decay problems of the antibody ELISA or any confounding factors such as concurrent infection or nutritional stress which could affect the host's antibody response to liver fluke infection. The coproantigen ELISA can detect ESA from as few as 1-2 flukes (Mezo *et al.* 2004) and reliably detect infections of 10 or more flukes (Charlier *et al.* 2008) from 6 weeks post infection. The coproantigen ELISA has been shown to be able to quantify the liver fluke infection in Chapter 2 of this thesis, determining the presence of production limiting fluke burdens.

A mathematical approach to estimating the test characteristics in the absence of a gold standard is through using Bayesian latent class modelling (Joseph *et al.* 1995; Rapsch *et al.* 2006; Mazeri *et al.* 2016). Bayesian latent class models (LCM) provide robust estimates of disease prevalence and test parameters including Se, Sp, aiding a better interpretation of assay characteristics. Furthermore, the use of Bayesian LCM allows for smaller sample sizes and the comparison of multiple, independent, dichotomous diagnostic tests to calculate the characteristics of each (Pouillot *et al.* 2002; Rapsch *et al.* 2006; Mazeri *et al.* 2016) in populations where the only known variable is the apparent disease prevalence. This method of analysis has been used in studies investigating disease prevalence and test characteristics where there is no gold standard, including the prevalence of liver damage caused by pithomycotoxicosis in cattle (Laven *et al.* 2020), liver fluke in Swedish cattle (Rapsch *et al.* 2006)

and *Strongyloides* infection in humans (Joseph *et al.* 1995). It has also been used for the estimation of diagnostic test characteristics (Horigan *et al.* 2011; Mazeri *et al.* 2016). There are a number of Bayesian LCM models available online, developed to assess single or multiple diagnostic tests in single or multiple populations.

Important requirements for using some Bayesian LCM are that the diagnostic tests are independent and the true prevalence of disease in the populations are quite different. Regarding test independence, the following assumptions were made; a FEC relies upon the presence of eggs while a coproantigen ELISA relies upon the present of fluke specific ESA in the faeces, so these two tests are determined as independent. The In-House ELISA used in Chapters 2, 4 and 5, detects antibodies produced in response to the presence of the unrefined ESA antigen of the liver fluke while the IDEXX antibody ELISA detected antibodies specific to the f2 component of the ESA, so these two tests are not independent and could not be used concurrently in some models.

The aim of this study is to evaluate three commercially available diagnostic tests and the In-House ELISA, using Bayesian LCM, for their suitability to diagnose *F. hepatica* infection in naturally acquired, chronically infected adult cattle on New Zealand farms.

6.2 Materials and Methods

6.2.1 Study Design

Two Bayesian latent class models (LCM); *3 tests 2 populations* and 2 *tests 1 population* were selected to analyse data from earlier studies in this thesis. For the *three test two population model,* Herds J and K (Chapter 5) were used and included a comparison of coproantigen ELISA, IDEXX serum antibody ELISA and faecal egg count (FEC). For the *two test one population model,* Herd A (Chapter 4) was used and included a comparison of coproantigen and In-House ELISA tests

6.2.2 Animal selection and sampling.

All cattle in this study were adult dairy cows on the West Coast of the South Island with samples collected from three herds (Herds A, J and K). For Herds J and K cows were sampled in the same week in April (autumn) 2021, as described in Section 5.2.3. For Herd A, cows were sampled in March (autumn) 2019 as described in Section 4.2.3.

Serum samples were processed as previously described. In brief, they were transferred to a refrigerator once a clot had formed and then stored at 4°C. Faecal samples (collected as described in Section 2.2.2) from the Herd J were stored chilled insulated portable bins with frozen pads changed each day. Faecal samples from Herd K were stored on the day of collection in the same manner, with all samples transported to the laboratory and transferred into a chiller at 4°C, 60 hours after collection for Herd J and 16 hours after collection for Herd K. Serum samples were also transported to the laboratory in chilled containers and placed in a chiller at 4°C on arrival. The following day, 5g of faeces was weighed into 70mL plastic containers and stored at 4°C, while the remaining faecal material in the original plastic container was stored at -20°C. On the same day, serum samples were centrifuged at 1,100 g for 15 minutes (Thermo Scientific, Heraeus Megafuge 40) with duplicate samples pipetted into 1.5mL eppendorf tubes and stored frozen at -20°C. Serum and faecal samples from Herd A were stored and processed in the same manner as those of Herds J and K after arrival at the laboratory, six days after sampling.

6.2.3 Faecal egg counts

As described in Section 4.2.6.1. Animals with any eggs detected were assigned a positive result.

6.2.4 Coproantigen ELISA

As described in Section 4.2.6.1, cows with a Value ≥0.014 were assigned a positive result.

6.2.5 IDEXX serum antibody ELISA

The IDEXX antibody ELISA (IDEXX Fasciolosis Verification, IDEXX Europe BV, Hoofdorp, The Netherlands) was conducted as per kit instructions (Section 3.1.9). Serum samples were refrigerated after collection and centrifuged within one week before being stored at -20^{0}C until analysis 9 weeks later when they were processed on the same day for Herds A and B. Cows with a calculated SP% ≥30 were assigned a positive result.

6.2.6 In-House antibody ELISA

As described in Section 4.2.7.3, cows with a ODR ≥0.42 were assigned a positive result.

6.2.7 Statistics

6.2.7.1 Estimation of parameters for the coproantigen ELISA, IDEXX ELISA and the FEC diagnostic tests

The model used for estimating the test characteristics of the coproantigen ELISA, IDEXX ELISA and the FEC was an adaption of the original Hui and Walter *2 tests 2 populations* model (Hui and Walter, 1980) to one analysing *3 tests 2 populations*. The Hui and Walter model makes three assumptions; 1) that the prevalence of disease is different between the two populations, 2) that the sensitivity and specificity of each diagnostic test is the same for each population, and 3) that the tests are conditionally independent. The structure of the *3 tests 2 populations* model means there are 14 degrees of freedom (seven in each population) and 8 parameters to estimate (3 Sensitivities, 3 Specificities and 2 prevalences) which makes the model solvable. The model was implemented in JAGS (Plummer, 2003), a software using Markov chain Monte Carlo (MCMC) simulations to construct posterior distributions for the analysis of Bayesian hierarchical models. JAGS was run within R (Version 4.2.1) (RStudio Team (2020). RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/) using the runjags package (Denwood 2016). The first 10,000 iterations were discarded as burn-in and the next 200,000 iterations were used to construct the posterior distributions.

The LCM model incorporates prior knowledge by specifying prior distributions for test sensitivity, specificity, and true disease prevalence. Prior distributions may be based on literature or expert opinion, but if no prior information is available, vague, uninformed priors are used instead. Probabilities of all the possible combinations of test outcomes conditional on the unknown disease status are specified using the Se and Sp of each test and the true prevalence (p) of liver fluke in each herd (Mazeri *et al.,* 2016). For example, the probability that all three diagnostic tests are positive for Herd A =

$$p1[1] < -pi1 * se1 * se2 * se3 + (1 - pi1) * (1 - sp1) * (1 - sp2) * (1 - sp3)$$

Where $p1[1]$ is the probability of all three tests being positive in Herd A, $pi1$ is the true prevalence of liver fluke in Herd A, $se1$, $se2$ and $se3$ are the sensitivity of the coproantigen test, the IDEXX ELISA and FEC respectively and $sp1$, $sp2$ and $sp3$ are the specificity of the coproantigen test, the IDEXX ELISA and FEC respectively.

For the *3 tests and 2 populations* model, animals can be positive or negative for each of the three diagnostic tests giving eight possible combinations of test results. For this LCM model uniformed, uniform priors were used for estimating the prevalence (Beta (1,1)) meaning that every possible value for prevalence is equally likely as there was no informed data on the possible prevalence, and for estimating the sensitivity and specificity of the diagnostic tests informed priors were used, following the method described by Joseph *et al.* (1995). Briefly, published estimates of the test characteristics for the coproantigen test, the IDEXX ELISA and FEC were first obtained through a literature search (Chapter 1, Section 9) and are summarised in Table 6.1. Then the median of these estimates was used as an approximation of the mean and dividing the range from the highest to the lowest test estimates by 4, gave an estimate of the standard deviation and then squaring this gave the variance.

$$mu < -median(data)$$

$$var < -((max(data) - min(data))/4)^\wedge 2$$

The alpha parameters of the beta distribution were then calculated using the following formula:

$$alpha = \left(\frac{(1 - mu)}{var} - \frac{1}{mu}\right) * mu^\wedge 2$$

The beta parameters of the beta distribution were then calculated using the following formula:

$$beta = alpha * \left(\frac{1}{mu} - 1\right)$$

Convergence of the Bayesian LCM MCMC was assessed by visual inspection of the trace plots using the R Package coda (Plummer *et al.* 2006) along with examining the results of the Gelman and Rubin test and the Heidelberger-Welch diagnostic test.

This model is shown as model 1 in Appendix 2, Supplementary materials, Chapter 6. Models.

Table 6.1 Studies referenced for IDEXX antibody ELISA, coproantigen ELISA and faecal egg count sensitivity and specificity results to calculate the beta prior distributions.

IDEXX antibody ELISA studies	Sensitivity	Specificity
(Hutchinson 2003)	99%	95%
(Charlier *et al.* 2008)	82%	80%
(Charlier *et al.* 2008)	95%	88%
(Rapsch *et al.* 2006)	92%	94%
(Molloy *et al.* 2005)	98.2%	98.3%
Coproantigen ELISA studies	Sensitivity	Specificity
(Kelley *et al.* 2021b)	100%	not determined
(Palmer *et al.* 2014)	87%	99%
(Charlier et al., 2008) spring	98%	92%
(Charlier et al., 2008) autumn	90%	94%
(Mazeri et al., 2016)	77%	99%
FEC studies	Sensitivity	Specificity
(Kelley *et al.* 2021b) 2g faeces	88%	not determined
(Boray 1969)	30%	not determined
(Rapsch *et al.* 2006) 10g faeces	69%	not determined
(Anderson et al., 1999) 5g faeces	67%	100%
(Charlier *et al.* 2008) 4g faeces	42%	100%
(Charlier *et al.* 2008) 10g faeces	63%	96%
(Mazeri et al., 2016) 5g summer	81%	99%
(Mazeri et al., 2016) 5g winter	77%	99%
(Mazeri et al., 2016) 5g autumn	58%	99%

6.2.7.2 Estimation of parameters for In-House ELISA diagnostic test and coproantigen ELISA

The model used to estimate the test characteristics of the In-House ELISA and coproantigen ELISA is also an adaptation of the Hui and Walter *2 tests 2 populations* model to one analysing *2 tests 1 population* model. One herd (A) was used where cows were both serum and faecal sampled.

The Bayesian LCM was built using informed priors for the sensitivity and specificity of the coproantigen test based on the results from the *3 tests 2 populations* model and using a uniform, uninformed prior for the prevalence and the Se and Sp of the In-House ELISA test. The priors for the coproantigen test were calculated using the betaExpert function from the prevalence package (Rahman *et al.* 2019) using the mean and the lower 95% credible limit from the 3 tests 2 populations model output (Table 6.2). This model is shown as model 2 in Appendix 2, Supplementary materials, Chapter 6. Models.

Table 6.2 Informed beta prior distributions calculated using the betaExpert function and used for each diagnostic test in the Bayesian LCM

Test	Sensitivity	Specificity
IDEXX antibody ELISA	Beta (24.0,1.3)	Beta (24.4,1.6)
Coproantigen ELISA	Beta (23.6,2.6)	Beta (105.5,3.8)
FEC	Beta (6.4,3.1)	Beta (97,1)

6.3 Results

6.3.1 *3 tests 2 populations* model

There were 109 cows from Herd J and 99 cows from Herd K sampled in the autumn of 2021 used in the model. The observed frequency of each possible combination of diagnostic tests results for Herds J and K, are shown in Table 6.3.

Table 6.3 The observed frequency of each possible combination of coproantigen ELISA, IDEXX antibody ELISA and faecal egg count positive and negative diagnostic test results for Herds J and K.

Coproantigen ELISA	IDEXX antibody ELISA	Faecal egg count	Frequency Herd J	Frequency Herd K
Positive	Positive	Positive	10	8
Positive	Negative	Negative	44	46
Negative	Positive	Negative	6	0
Negative	Negative	Positive	7	0
Positive	Positive	Negative	9	34
Positive	Negative	Positive	11	10
Negative	Positive	Positive	0	0
Negative	Negative	Negative	22	1

The results of the Bayesian LCM for 3 tests 2 populations are shown in Table 6.4, and the diagnostics for the model were satisfactory. The trace plots for each of the estimated parameters showed that MCMC algorithm had converged to a stationary distribution (Figure 6.1). The Gelman and Rubin point estimate was 1 for all the estimated parameters and the model also passed the Heidelberger-Welch convergence test.

The calculated liver fluke prevalence of Herd J was 71% (95% CI 60-82) and much higher in Herd K at 99% (95% CI 96-100) The calculated Se and Sp for the coproantigen ELISA were 96% (95% CI 92-100) and 96% (95% CI 92-99), IDEXX ELISA 43% (95% CI 36-50) and 90% (95% CI81-98), FEC 26% (95% CI 19-32) and 95% ((95% CI 91-100) respectively (Table 6.4).

Table 6.4 Results of Bayesian Latent Class 3 tests 2 populations *model showing the mean and the 95% Bayesian credible limits for the true prevalence of disease in Herd J and Herd K, and for the test properties of Se and Sp for the coproantigen ELISA test, the IDEXX antibody ELISA and the FEC.*

	Mean	Lower credible limit	Upper credible limit
Prevalence Herd J	71%	59%	83%
Prevalence Herd K	99%	96%	100%
Sensitivity coproantigen ELISA	96%	92%	100%
Sensitivity IDEXX antibody ELISA	43%	36%	50%
Sensitivity FEC	26%	19%	32%
Specificity coproantigen ELISA	96%	92%	99%
Specificity IDEXX antibody ELISA	90%	81%	98%
Specificity FEC	95%	91%	100%

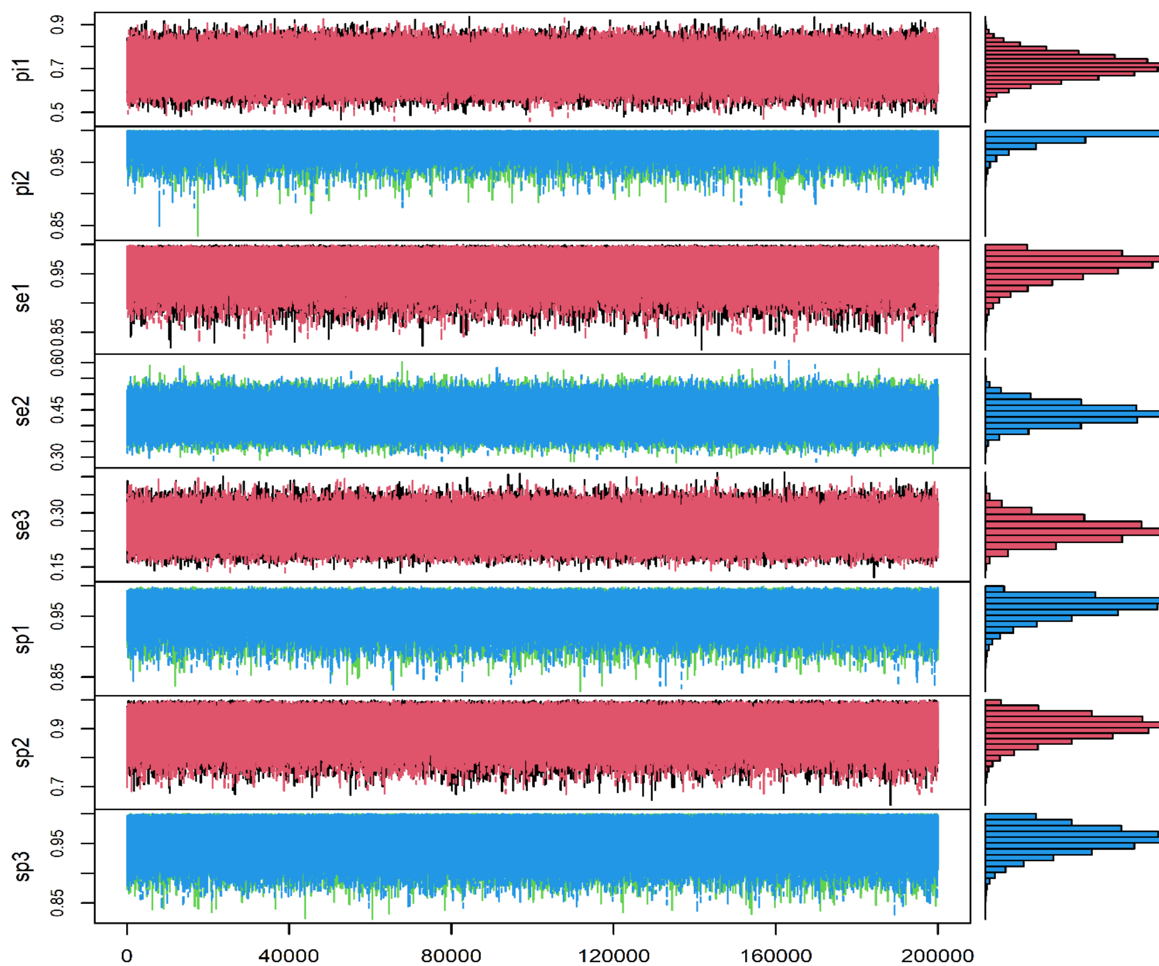

Figure 6.1 Trace plots for Bayesian LCM model, pi1 is the prevalence of liver fluke infection on Herd J, pi2 is the prevalence of liver fluke on Herd K, se1 and sp1 is the sensitivity and specificity of the coproantigen test, se2 and sp2 is the sensitivity and specificity of the IDEXX ELISA and se3 and sp3 is the sensitivity and specificity of the FEC test.

6.3.2 *2 tests 1 population model*

There were 85 cows with coproantigen and In-House ELISA results, and the observed frequency of diagnostic test result combinations for Herd A are shown in Table 6.5. The priors used for the coproantigen ELISA test, calculated from model1 results, were beta (81, 3.4) for sensitivity and beta (81, 3.4) for specificity.

Table 6.5 Each possible combination of coproantigen ELISA and In-House antibody ELISA positive and negative test results and the observed frequency of each for Herd A.

Coproantigen ELISA	In-House antibody ELISA	Frequency Herd A
Positive	Positive	24
Positive	Negative	28
Negative	Positive	11
Negative	Negative	22

The performance of the diagnostic tests is shown in Table 6.6; the In-House ELISA has a calculated sensitivity of 47% (95% CI 33 – 60) and a specificity of 67% (95 CI 50– 83) while the coproantigen ELISA has a calculated sensitivity of 96% (95% CI 92-99) and a specificity of 96% (95 CI 92-99. The calculated liver fluke prevalence of Herd A was moderate at 62% (95% CI 50-73).

Table 6.6 Results of Bayesian Latent Class model showing the mean and the 95% Bayesian credible limits for the true prevalence of disease in Herd A for the test properties of the coproantigen ELISA and In-House antibody ELISA tests.

	Mean	Lower95	Upper95
Prevalence of liver fluke	62%	50%	73%
Sensitivity of coproantigen	96%	92%	99%
Sensitivity of in-house ELISA	47%	33%	60%
Specificity of coproantigen	96%	92%	99%
Specificity of In-House ELISA	67%	50%	83%

The trace plots for each of the estimated parameters showed that MCMC algorithm had converged to a stationary distribution for the In-House ELISA model. The Gelman and Rubin point estimate was 1 for all the estimated parameters and the model also passed the Heidelberger-Welch convergence test.

6.4 Discussion

This study used Bayesian LCM to estimate the test characteristics of diagnostic tests when there was no gold standard test available with the aim of identifying the suitability of these diagnostics tests for use in New Zealand cattle. The coproantigen ELISA has Se 96% and Sp 96% (in both models), IDEXX antibody ELISA had Se of 43% and Sp 90%, In-House antibody Se 47% and Sp 67% and FEC Se 26% and Sp 95%.

The sensitivity and specificity values estimated for the coproantigen ELISA from the 3 tests 2 populations model were close to published studies (Table 6.1) whereas the results for the IDEXX ELISA and the FEC had far lower sensitivities, but more comparable specificities to published results (Table 6.1). The In-House ELISA, since it used an adapted methodology, had no literature to compare it to,

however the results were poorer than the IDEXX ELISA. The lactating dairy cows in this study were all sampled at a similar calendar date to avoid any seasonal influence on test characteristics (Charlier *et al.* 2008) and when infection prevalence in New Zealand is predicted to be at a peak (autumn).

6.4.1 In-House ELISA

In Chapter 2, the In-House ELISA was calculated to have Se 67% and Sp 80%, both much higher than the figures calculated in the models used in this chapter (47% and 67% respectively). In Chapter 5, the IDEXX antibody ELISA was used as the gold standard test and the comparison of those results with this study support the argument that an imperfect gold standard test will over or underestimate the Se and Sp to reflect itself. This creates confusion for a veterinarian when the advertised Se and Sp of tests often do not reference the gold standard test or the disease prevalence of the study population. If the new diagnostic tool is designed to determine current infection and an antibody detection ELISA is used as the gold standard test, then the new test is compared to the hosts response to historic fluke infection, not necessarily the current fluke burden. The unreliability of a host antibody titre to reflect the current fluke burden has been a recurring theme in this thesis and is reinforced once more. The dynamics of the immune response and antibody decay is complex and difficult to study in animals with naturally acquired, chronic infections. The half-life of IgG1 and IgG2 are similar to those published for protozoal or viral infections, for example against *Neospora* was estimated to be 19.6 ± 5.2 days (Hietala and Thurmond 1999) being 13 and 20 days respectively (Levieux *et al.* 1992a) which could mean that animals continue to test positive for liver fluke for several months after active infection has been cleared.

The poor performance of the In-House ELISA also reflects the difficulties of develop a diagnostic test for liver fluke using unrefined ESA based on a published protocol. It was certainly disappointing to have this test perform very poorly. The Bayesian LCM results obtained are little better than a coin toss and so it is unlikely there will be any commercial interest in this test. Interestingly, the sensitivity for the In-House ELISA of 43% (95% CI 36 – 50) was very similar to that for the IDEXX ELISA 47% (95% CI 33 – 60). However, the specificity was lower at 67% (95% CI 51 – 83) for the In-House ELISA versus 90% (95% CI 81 – 98) for the IDEXX ELISA.

6.4.2 Value of using Bayesian LCM

When any imperfect diagnostic test is used as the gold standard for determining the test characteristics of a second test, the results can be over or underestimated and biased toward the imperfect gold standard test (Molloy *et al.* 2005; Salimi-Bejestani *et al.* 2007; Munita 2019). This chapter supports the use of Bayesian LCM to estimate the disease prevalence and test characteristics when the use of total fluke counts (the gold standard test) is not practicable, as will often be the situation when a test is being assessed in naturally infected animals. Using data from prior test Se and Sp and input of experts or published results, the beta distribution priors can be calculated for use in one of the many LCM models. The Bayesian LCM are a valuable tool when the populations have different disease prevalence, test characteristics do not change between populations and the diagnostic tests are independent, with many models available to use on the internet, having user-friendly interfaces, instructions and diagnostics. In situations where an imperfect gold standard test is used as a reference test, the use of Bayesian LCM to calculate test characteristics is recommended.

6.4.3 Which diagnostic test to use?

When choosing a diagnostic test to use, the veterinarian must first ask themselves what they are trying to determine. The wide-ranging diagnostic test characteristics estimated in this study reinforces the limitations of using some diagnostic tests in an individual at one time point and the benefit of using tests in parallel (George 2019, Sekiya 2013).

The coproantigen ELISA was the most reliable assay for both confirming infection and freedom from infection, with both a high sensitivity 96% (95 CI 92- 100) and specificity 96% (95% CI 92 - 99) in the *3 tests 2 populations* model and 96% (95% CI 92 - 99) and specificity 96% (95% CI 92 - 99) in the *2 test 1 population* model. Interestingly, these values are very similar in both models. This assay detects ESA released from the flukes, so the presence of flukes within the biliary system enables the transit of these compounds to the intestinal system where they are mixed with the digesta to be detected in faeces. This test does not rely on a host response and the performance of the test indicates that it could almost be used as a gold standard in live animals. This is very exciting and means clinicians finally have access to a reliable test. While the coproantigen ELISA requires expensive, specialist equipment found in commercial and research laboratories, the commercial cost of running the ELISA is likely to be similar to the FEC due to the ability to process multiple samples. The student took 23 hours to prepare and count 100 FEC while analysis of 237 samples using the coproantigen test took a total of 14 hours. The speed of processing multiple samples and very high Se and Sp make it a test of choice to indicate current infection status for determining appropriate treatment or management decisions. A review of the literature has found that cattle faecal samples can be frozen before coproantigen analysis but the length of time they are kept frozen is not reported in many studies. For example, sheep samples were stored frozen for 12 weeks (Flanagan *et al.,* 2011) and 17 weeks in humans without impacting the optical density (Ubiera *et al.* 2009). In Chapter 2 and 4 of this thesis, faecal samples were stored frozen for 26 and 4 months respectively with the samples from Chapter 2 having the highest values, indicating that the antigens were not greatly reduced by this prolonged period of freezing.

The FEC is useful in veterinary clinics with access to limited diagnostic tools. If infection is suspected the presence of any eggs confirms infection. A production limiting infection of 10 or more flukes can be identified by the presence of one or more eggs in a 10g sample (Mezo *et al.* 2010b), but in another study, Charlier *et al.* (2008) determined that the detection of one egg in a 4g sample of faeces was 10.7 more likely to indicate infection at a level likely to cause production losses compared to a sample with no eggs detected, and 2.5 times more likely using a 10g faecal sample. The use of repeated counts from the same sample also increases Se (Rapsch *et al.* 2006) making this a useful, if somewhat laborious, diagnostic method.

The reasonable specificity of the IDEXX ELISA does mean that this test continues to have a place in the diagnostic toolbox as an indicator of past infection. The test results used here were for the individual animal but if a similar specificity was found for the bulk milk test, then a positive result should give good confidence that a herd is infected, unfortunately the low sensitivity means that a negative result is not informative. The bulk milk test was shown to be highly repeatable with herds sampled in consecutive weeks (Chapter 3) so serial sampling throughout the lactation may provide more useful information around the dynamics of infection in the herd. We suggest that if a farm test is negative but there is high suspicion that liver fluke is present then a coproantigen test should be run for a random sample of 10+ cows.

6.4.4 Summary

In conclusion, the coproantigen ELISA test performed very well under NZ field conditions and should be adopted as the gold standard for diagnosing liver fluke infection in NZ herds.

All three herds had a high estimated prevalence of liver fluke in the sampled cows with Herd K having almost 100% of cows infected, reflecting how common liver fluke is on the West Coast and why sustainable solutions are needed.

A limitation of this study is that herds were only sampled in autumn. Seasonal effects on diagnostic tests have been reported (Charlier *et al.* 2008) and need to be investigated further to determine if this effect is also present in New Zealand where animals are not housed so may be ingesting metacercariae in winter. One herd (K) also had very high predicted prevalence which may affect the model outputs, although all trace plots were within desired parameters.

Future work should aim at serial faecal, serum and bulk milk analysis of herds throughout a lactation to investigate the impact of season on test characteristics and investigating the performance of the coproantigen ELISA test on bulk faeces in New Zealand farming systems.

6.5 Ethics permission

This study was performed under the approval of Massey University Animal Ethics Committee, Protocol MUAEC 21/06

6.6 Supplementary Materials

Supplementary materials for Chapter 6 are contained in Section 3.2.6.

Chapter 7. General Discussion

The overarching aim of this thesis was to investigate the impact of liver fluke infection on milk production in dairy cattle. It was apparent that there was a small, but repeatable effect on MF% seen in studies in both Chapters 4 and 5. In the cross-sectional study of Chapter 4 there was a significant negative linear relationship between IDEXX SP% and MF%, but not MP%, ML%, MS, or ECM. Although the magnitude of the impact was small, with MF% decreasing 0.0004 % points for every increase of SP%, a similar trend was also confirmed in the longitudinal study in Chapter 5 where cows that were *negative* and seroconverted to *strong positive* over the lactation had a MF% 0.22 %points lower than in cows that remained *negative*, with an economic impact of $55.19 for an infected cow compared to an uninfected cow. Few other comparable studies have been undertaken. Kostenberger et al. (2017) compared 'slight' antibody positive and 'strong positive' farms and observed that MF% changed from 4.192% down to 4.1% which is a smaller drop than the 0.22 percentage point reduction observed in the study in Chapter 5. A Belgian study (Charlier *et al*. 2007) also only found a relationship between cows in the 75th percentile of results compared to cows in the 25th percentile with a decreased MF% of 0.06%. Overall this effect has both animal health and subsequent economic effects. The greater the herd prevalence of infection, the greater the economic impact with the calculated impact likely to be an underestimate as it does not take prior infections and thus potential liver pathology into account. A conundrum for farmers and their veterinarians is to decide whether to treat or otherwise control liver fluke infections.

7.1 Prevalence of liver fluke infection

It is certainly evident that liver fluke is a common parasitic infection in dairy herds on the West Coast of the South Island. However, the total fluke burden in each cow appears to be low as indicated by the coproantigen values from cows sampled in three herds to determine fluke burden, based on the formula from Section 2.3.5.2. This potentially conflicts with the results from the herd BME survey (Chapter 2) which suggested that approximately 50% herds had a production limiting infection, so the BME may be providing an overestimation of the production impact in at least some herds. The prevalence of liver fluke in dairy herds on the West Coast of the South Island in both the autumn and spring showed regional clustering of herds with IDEXX ELISA values indicating *strong positive* and *no or very weak* herds (Figures 3.2 and 3.3). The inter-class correlation of IDEXX SP% calculated from 1538 cows in 11 herds in Section 4.3.8 showed 40% of the variance seen was between herds, whereas a similar calculation in Belgium (Charlier *et al.* 2012) indicated the between herd variation was only 7%. This indicates the risk of fasciolosis is highly variable on the West Coast dependant on local conditions, so an effective liver fluke risk forecasting tool needs to be able to calculate the risk for smaller geographical areas.

7.2 Liver fluke risk prediction models

Models using air temperature, rainfall, evapotranspiration and number of wet days have been developed and validated overseas to predict the risk of infection with *F. hepatica*, usually in monthly increments (Ollerenshaw and Rowlands 1959; Malone *et al.* 1987; McCann *et al.* 2010). This then estimates the risk of fascioliasis. In New Zealand, NIWA have virtual climate stations (VCS) distributed on a 5km^2 grid over the country estimating the weather at each by interpolating data from actual weather stations. Data from these VCS was used by Haydock *et al.* (2016) to predict future liver fluke risk in New Zealand based on a model developed by Malone *et al.* (1998). The liver fluke prevalence data reported in the current studies could be used to validate Haydock's model, and if successful the

model could then be used in the development of risk profile reporting for farmers to make informed decisions about liver fluke risk and alter herd management practices where appropriate. The geographical variation between effectively river valley regions needs further exploring to determine the reason as this may provide some answers for developing control options.

7.3 Interpreting diagnostic tests

The use of Bayesian LCM to calculate test characteristics when there is no suitable gold standard test was shown to be able to characterise infection status in Chapter 6 with the coproantigen ELISA having the highest sensitivity (Se) and specificity (Sp). This and the significant relationship between fluke counts and coproantigen value indicates that this test could be a very useful indicator of fluke burden in live animals and be the gold standard test for assessing the characteristics of any new tests, rather than having to rely on slaughtering animals for total fluke counts.

The host antibody concentration as determined by serum IDEXX ELISA analysis was shown to be highly variable within herds (Section 4.3.8), a result supported by Charlier (2012). This degree of variation within a cohort of animals being managed as a single group indicates the complexity of the individual host immunoreactivity to the presence of the parasite. The slow decay of antibodies after successful treatment or self-cure also allows an animal to test positive even when not infected. Indeed, this factor overlies many of the results from these studies where there is uncertainty as to reinfection or just slow decay of antibody levels.

The IDEXX ELISA is a good test for detecting the historic presence of liver fluke infection, either in the individual or a pool of sampled animals and was shown to be reliable when herds were analysed twice in Section 3.3.1, one to two weeks apart. However, it was not able to determine the number of flukes present as was evidenced in Chapter 2 where all animals had a *positive* or *strong positive* result even if no flukes were detected in some animals. However, the calculated sensitivity (Se) of the IDEXX antibody ELISA in Table 6.4 was much lower than published data. The higher specificity (Sp) of the IDEXX antibody ELISA makes this test a good indicator of past infection, particularly at the group or herd level. The tendency to over interpret the SP% of this assay in individual cows needs to be tempered as was also suggested by Takeuchi-Storm (2021), and with very few cows in the current studies in the *mild positive* and *positive* diagnostic categories (Tables 4.7, 5.4), the dichotomous categories of negative and positive may be more appropriate. However, in the herd bulk milk ELISA there was a better distribution of SP%, with herds also in the *low positive* and *medium positive* categories (Tables 3.4, 3.6) so here the use of the four diagnostic categories may be more appropriate.

The In-House ELISA was based on the method developed by Salimi-Bejestani (Salimi-Bejestani *et al.* 2005), using an unrefined excretory secretory antigen. This assay was used in an attempt to reduce costs but unfortunately was determined to be a poor diagnostic tool and consequently replaced in all studies with the IDEXX ELISA.

The coproantigen ELISA performed very well in these current studies and was the diagnostic test of choice to determine the current fluke burden in chronic, naturally acquired infections in cattle. The linear relationship between coproantigen value and total fluke count in Section 2.3.5.2 indicated that this test reliably quantified the fluke burden, although the sample size was small (n=37). Nevertheless, the resulting equation now provides the opportunity to estimate actual fluke burdens in cattle which will be a useful starting point for rural veterinarians. This test could be used to determine if the fluke burden was sufficient to be likely causing production losses and thus treatment is economically beneficial, with coproantigen value (Val) ≥17.5 indicating an infection of 10 or more flukes and Val of

≥37 indicating a burden of 30 or more flukes. The cattle with more flukes were also of lower body weight at slaughter than their herd mates with fewer, or no flukes present, a result supported by Mazeri (2017). This indicates that liveweight gain was reflected by the current fluke burden, which may not always be the case.

The impact of season on the test characteristics requires further evaluation with liver fluke diagnostics. The data provided in this study (Section 5.3.7) and by others (Charlier *et al.* 2008) is too compelling to allow the assumption that diagnostic tests perform equally throughout the year. The variation could be due to parasite, host, environmental factors or just overinterpretation of the ELISA result. These are all factors that required further investigation. Understanding any impact of season of testing on the characteristics on the coproantigen ELISA would also support the use of this assay at the best choice of the diagnostic tests. Nevertheless, the coproantigen ELISA appears to be a good test for quantifying liver fluke infection in live animals. If this result is supported by further work then this test can be used to validate other diagnostic tests including the bulk milk ELISA, further determine the impact of the current fluke burden on liveweight gain, and investigate relationships with milk production.

The IDEXX BME test has been shown to be highly repeatable with an *almost perfect* kappa analysis in herds sampled in consecutive weeks in Chapter 3, but accuracy was not analysed. To determine the value of anthelmintic treatment of the herd against liver fluke, an accurate estimate of the within-herd prevalence is desired. Correlating the results of coproantigen analysis of a sub-set of cows within herds against the result of the bulk milk ELISA would be valuable to determine the accuracy of the quantification analysis of the calculated bulk milk SP%.

7.4 Change of faecal egg counting methodology from 10g to 5g faecal weight

In Chapter 2, 10g of faeces was used for faecal egg count (FEC) analysis while for Chapter 4, a 5g sample was used. The change was made to allow for sufficient weight of faeces to be available for both FEC and coproantigen analysis. The faecal samples from the West Coast were collected in the autumn when milk volume per cow is lower, thus the speed of the rotary platform can be increased to process more cows per hour. This increased speed allows less time to sample each cow, making it more difficult to collect a full pottle of faeces. To ensure there was sufficient faeces for both analyses, 5g was chosen after reviewing recent published studies (Section 1.11.5). This change in methodology prevented the comparison of the results between Chapter 2 and 4.

Studies have shown that in sheep faecal samples spiked with eggs, the sedimentation FEC method would identify approximately one third of what was added. The study has not been repeated in cattle, but the student's personal experience is that sheep faeces contain more small diameter plant material particles which made identification of eggs more difficult compared to cattle samples. This could be due to the cud chewing and rumination process in sheep breaking the plant material into smaller pieces. It would be useful to study the egg recovery rate for cattle faeces. Regardless of this, the detection of any eggs in a 4g faecal sample is 10.7 times more likely to identify an animal with a fluke burden ≥10 than a sample where no eggs were detected. The detection of only one egg in chronically infected cattle is likely to be indicative of a production limiting infection.

7.5 Analysing the impact of the effects of liver fluke infection over a lactation based on a single sample near the end of lactation

In Chapter 4 the liver fluke infection of cows was determined using an antibody detection ELISA near the end of that lactation of either 20-40% of cows in the herd (8/11 herds) or 91-100% (3/11 herds). As discussed in Section 4.4.2 this antibody concentration determination is a historic report of liver fluke infection, requiring the cow's immune system to react to the presence of the parasite, and does not quantify the number of flukes present. A further complexity was that cows may have been infected since any last effective treatment. Such treatment, if any, will have been before calving due to milk withholding periods which was at least ten months prior to testing. This single assessment was unable to determine any associations between infection status and milk production parameters in cows that were likely to have been exposed to the parasite over prolonged periods.

7.6 Lack of milk production data

The inability to assess milk production parameters more than four times over a lactation of 271 days (Table 1.2), and also with 2/4 herds only having three herd tests in the longitudinal study hinders the ability to detect associations between liver fluke infection and changes in milk constituents. The collection of milk from individual cows for analysis requires specialist monitoring equipment not present on most dairy farms in New Zealand, so only occurs when trained personnel perform the milk sampling, at a cost to the farmer. In some overseas studies, milk can be analysed on a daily basis, strengthening the dataset. In spite of this, there was an association between cows that were *negative* at the first sampling point and *strong positive* at the second and a decrease of milk fat percentage.

7.7 Impact on Milk Fat %

The negative impact on MF% for each *uninfected* cow that became *infected* resulted in a loss of $55.19 and requires further investigation to confirm this cost. This impact on milk production could also be used as a proxy for the welfare costs to the infected cows. Determining if a liver fluke burden is causing production losses now or in the future is of more relevance to farmers and their veterinarians than just diagnosing its presence. Measuring liver damage at the commencement of lactation, serial sampling of cattle through the lactation using the coproantigen ELISA to better quantify and determine time of infection and increasing the number of herd tests conducted may all aid to better quantify the cost of infection on dairy farm.

7.8 Impact of treatment on milk production not studied

Due to drenches with efficacy against liver fluke having milk withholding periods, the impact of treatment of infected cows could not be tested. Overseas studies have investigated the treatment of cows at the end of one lactation on milk production in the subsequent lactation (Charlier *et al.* 2012; Kostenberger *et al.* 2017; Novobilsky *et al.* 2020) with the result including increased milk volume and milk protein percent. Treatment of cows at the end of lactation is a common practice on the West Coast with 69% of survey respondents indicating they drenched with a product specifically targeting liver fluke (Section 3.3.6). There was a strong association between the autumn bulk milk ELISA result and drenching cows (Section 3.3.7) where herds with a *strong* result were more likely to be treated than herds with a *no or very weak* result. For herds with either a *medium* or *strong* result in the autumn, indicating that 20% or more cows are infected, the decrease in SP% was no greater in the spring for herds that drenched (n=75) compared to those not drenched (n=16). Three possible causes

of the drench appearing to have little impact on the infection status of the herd in the spring as compared to no treatment, include ineffective treatment, rate of antibody decay and subsequent reinfection. No attempt was made to determine which products farmers used to drench cows or whether the treatments were effective. Regarding the rate of antibody decay, there were seven months between the two sampling points (March and October) which should be sufficient to see a drop in SP% (Munita 2019), so if treatment was effective this can be discounted. Cows are not housed over the winter, rather they remain grazing a mixture of pasture, silage and forage crops over the dry period on the same farm grazed during lactation, or at a different location. This potential for reinfection soon after treatment may hinder the positive impact of any treatment on milk production but needs to be investigated, as the removal of the current fluke burden may still be beneficial, but it does not appear to impact the eventual reinfection of cows. If funding had been available, an analysis of serum GGT to indicate choloangiohepatic pathology could have be useful to investigate the impact of repeated liver fluke infection over the years in older cattle.

7.9 Further research

Future research on liver fluke in New Zealand should aim to understand the epidemiology of the parasite including the intermediate host snails, both on the West Coast and the other areas of New Zealand such as Northland, where fluke occurs. Developing effective control methods does need this more detailed information. At present, liver fluke infection is not considered to be overly important in general but many farmers are aware this is not correct and over the whole country the impact of liver fluke is likely to be underestimated. Both the effectiveness of the practice of treating cows at the end of lactation to improve animal health and performance, and the efficacy of products used requires investigation.

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3. Appendices

3.1 Standard Operating Procedures

3.1.1 Standard Operating Procedure 1: Identification and counting Fasciola hepatica eggs per gram of faeces.

Purpose

To identify and count the number of *Fasciola hepatica* eggs in faecal samples as a diagnostic tool to qualify and quantify infection. The counting system relies on fasciola eggs being recovered from a faecal sample of a set weight, identified and counted where each egg represents one egg per weight of faeces which is calculated to eggs per gram.

Materials and Equipment

- Workbook to record results
- Electronic scales to weigh faeces (accuracy ±0.1g)
- Small sieve (tea strainer)
- Small round bowl of approximately 100ml capacity
- Teaspoon
- Water
- Flukefinder® or parasitological sieve with mesh ≤53um
- Wash bottle of 250ml capacity
- Vertical cylinder, 100ml capacity
- Suction device
- Falcon tube of 15mm capacity
- Petri dish with etched base for identification and counting of eggs
- Methylene blue 0.5%
- Microscope with 40x magnification

Procedure

a) Soft faeces are stirred using a spoon to mix the contents

b) A small sieve and spoon are placed in the small round bowl on the electronic scales.

c) Tare the scales to 0.0kg.

d) The desired weigh of faeces are transferred into the sieve.

e) Water is added to cover the faeces.

f) Soft faeces are mixed by pressing the back of the spoon against the sieve of the tea strainer while immersed in the water.

g) Hard pelleted faeces are left to soak until they are able to be crushed and mixed using the spoon.

h) The faeces are mixed and the tea strainer raised above the water to allow all liquid and faeces to drain into the bowl. The faecal material in the tea strainer is discarded.

i) The liquid in the bowl is poured into the Flukefinder assisted by the wash bottle.
j) Tap water is added to the Flukefinder to half fill the upper chamber.
k) The Flukefinder is tapped against the ball of your hand to assist the transfer of liquid through the mesh, emptying the fluid from the upper chamber. The chamber is half filled with water and this process is repeated until a total of 250ml of water has been used.
l) The two chambers of the Flukefinder are separated and the lower chamber placed upside down in the round bowl. Use the wash bottle to flush the flocculant from the sieve while gently rotating the Flukefinder to ensure all material is removed.
m) Pour this flocculant into the vertical cylinder and fill to 100ml with water. Stand for five minutes.
n) Suction the water from the cylinder to leave 5mm remaining. Take care not to suction the flocculant at the bottom of the cylinder by holding the suction device just below the surface of the water. Repeat this process two more times.
o) Pour the remaining flocculant into the falcon tube to be stored refrigerated for up to 34 days with only minor loss of bile staining.
p) Six samples can be prepared in this manner one hour (60 minutes).
q) Flocculant is agitated and poured into the petri dish and one drop of methylene blue added (the methylene blue adheres to the plant material and may assist with identification if Fasciola eggs although this step is optional).
r) Eggs are identified and counted
s) Eggs per gram (epg) is calculated using the formula

epg = total number of eggs / total weight of faeces

t) All equipment is thoroughly cleaned with water.
u) All faecal material is disposed in the hazardous waste bin.

3.1.2 Standard Operating Procedure 2: Collection and examination of livers and gall bladders at a slaughter house, visual scoring of livers.

Purpose

Describing the process of collection of livers and gall bladders from a slaughter house and assessment of these livers in respect to pathology caused by *Fasciola hepatica*.

Materials and Equipment

- Unique identification numbers for each animal in triplicate (carcass, liver and gall bladder).

- Plastic bags 60*60cm for holding liver samples, two per liver.
- Plastic bags 30*20cm for holding gall bladders, two per animal.
- Rubber bands to occlude plastic bags, four per animal.
- Rubber bands to occlude major bile ducts, two per animal.
- Plastic tray to hold viscera, one per animal.
- Chilled containers for transport of livers to laboratory for examination.
- Chiller for storage of livers and gall bladders in laboratory
- Sharp knife

Procedure

a) Contact slaughter house to arrange permission to collect the organs.

b) Each animal is given a unique identifier number on entry into the slaughter house.

c) The animal is weighed prior to slaughter.

d) The internal organs including the liver are eviscerated and placed in a plastic tray with the same identifier number the animal was assigned.

e) The liver and gall bladder are removed from the other organs using a sharp knife.

f) The gall bladder is removed and the common bile duct occluded using two rubber bands. The gall bladder is paled inside two plastic bags along with the slaughterhouse unique identification,

g) The liver from each animal is removed and doubled bagged containing the slaughterhouse unique identification.

h) The livers and gall bladders are transported 3 hours to the University for either immediate examination or chilling overnight.

i) Livers are weighed on scales accurate to 0.01kg

j) Livers are visually scored using the method described by Sargent (Sargent *et al.* 2009)

Liver Score	Pathology description
0	Absolutely no pathology evident – liver normal colour, consistency and no visible sign of fluke lesions
1	Small areas of scar tissue and lesions, <5% of liver affected

2	Moderate areas of scar tissue and lesions present, occurring in 5-10% of the liver
3	Moderate areas of scar tissue, thickening of the bile ducts, small to moderate areas of necrosis, pus, 10-20% of liver affected
4	Moderate to large areas of scar tissue, “pipe stem” liver starting to occur. Moderate areas of necrosis pus, haemorrhage, 20-30% of liver affected
5	Large areas of scar tissue, and pipe stem liver. Multiple necrotic foci, pus, haemorrhage, severe degeneration and >30% of liver affected

k) Livers are photographed

l) Gall bladders are removed from the plastic bags onto a plastic tray and incised, inverted and examined for the presence of liver flukes.

3.1.3 Standard Operating Procedure 3: Collection and preparation of excretory – secretory materials from adult Fasciola hepatica

Purpose

Describing the process of collection, concentration and determination of proteins present in the excretory – secretory (ES) products from *Fasciola hepatica* for use in ELISA diagnostic assays.

Materials and Equipment

- Sheep identified with faecal egg count as being infected with adult liver flukes.
- Captive bolt humane euthanasia gun.
- Sharp knife
- Examination gloves.
- Falcon tubes, 50ml
- Eppendorf protein low binding 0.5ml vials
- PBS pH 7.4 (phosphate buffered saline), Thermo Fisher Scientific, 1000ml warmed to 37C
- RPMI Medium 1640 (Gibco by Life Technologies Corporation), 500ml warmed to 37C
- Deionised water
- Penicillin Streptomycin 100x
- cOmplete mini, EDTA-free Protease Inhibitor Cocktail (Roche ##) tablet
- Plastic tray 40*30cm with raised edges to hold livers and prevent flukes washing away while being harvested.
- Plastic culture 12 well plates (Cellstar 12 well culture plate, Greiner Bio-One) to hold flukes in RPMI solution overnight

- Plastic pipettes
- Vivaspin 20, 10000 concentrating chambers
- Pierce BCA Protein Assay Kit (Thermo Fisher Scientific)
- Gel electrophoresis Criterion Precast gel, 10% Tris-HCl, 1.0mm, Bio-Rad Laboratories Inc, 2000 Alfred Nobel Drive, Hercules, CA 94547
- Centrifuge

Procedure

a) Liver fluke infected sheep euthansed and livers removed.

b) Liver placed on tray and major bile ducts incised to expose adult flukes.

c) Flukes placed into falcoln tubes containing PBS warmed to 37C

d) Flukes rinsed in warmed PBS six to eight times to remove blood and bile until PBS remained clear.

e) Add 2ml of warmed RPMI to each well of the plates.

f) Gently remove flukes from falcoln tubes to add three to each well of the plates.

g) Add RPMI to fill each of the wells and gently tip the plate to remove most of the RPMI without losing the flukes. Repeat this process three times.

h) A 2.5ml of Penicillin Streptomycin 100x to 250ml warmerd RPMI. Add sufficient of this solution to cover all of the flukes.

i) Incubate overnights at 37C, 5% CO_2.

j) The next day pipette supernantant from wells into 50ml Falcoln tubes.

k) Centrifuge the supernatant at 10,000g, 4C for 30 minutes at a rigid angle.

l) Pipette the supernatant intoclean 50ml Falcoln tubes leaving the bile salts in the centrifuged tubes.

m) Concetrate the supernatant in Vivaspin 20, 1000. Pippete supernatant into upper chamber and centrifuge 5000 rpm, 4C, 70 minutes, rigid angle. Flush the concetrated supernatant in the fliter chamber with deionised water to release larger protein stuck on fliter, pipette into Falclon tube .

n) Add one cOmplete tablet (antiprotease) to 1000ul of the concentrated supernatant collected from the flither chamber and mix well. Add 0.6ml of this to 30ml of the concentrated supernatant and mix.

o) Pipette concrentrated supernatant (fluke excretory – secretory antigens) into Eppendorfs and label, store at -80C.

p) Assess protein concentration with Pierce BCA kit

q) Gel electorphoresis of concentrated supernatant

3.1.4 Standard Operating Procedure 4: Total Fluke Counting procedure

Purpose

To quantify the number of *Fasciola hepatica* present in each liver to qualify and quantify the infection.

Materials and Equipment

- Examination gloves
- Carving knife 240mm
- Sharpening steel
- Large straight scissors
- Smaller straight or curved scissors (to fit inside the medium bile ducts)
- Rat tooth forceps
- 90ml pottle filled with water for fluke storage
- Wash bottle filled with water to flush bile ducts
- 1 litre water bottle for flushing sieve
- White plastic trays with lip (easier to see flukes on white background)
- Containers with 0.2m high sides to hold livers while soaking
- 250 mesh sieve
- Petri dishes
- Dissecting microscope 4X magnification
- Desk lamps
- 2 * 10 litre buckets
- Tap water (warm and cold)

Procedure

a) Frozen livers thawed 48-96 hours.

b) Livers taken from chiller and placed in the bags in a container with sides 0.2m.

c) Outer bag cut and inner bag containing liver tipped into container. Approximately 500ml of tap water added into the cut bag and the contents sluiced around and then tipped into the container. Bag discarded.

d) Inner bag cut and liver tipped into the container. Approximately 500ml of tap water used again to sluice this bag. This is done as flukes were attached to the plastic bags and this action would remove them.

e) A further 1-2 litres of warm water added to the containers to warm the livers to make it more comfortable when slicing them.

f) Liver removed from container and placed onto white plastic tray with a low lip to prevent any fluid escaping (as flukes could be lost).

g) The blood and water is poured through a 250 mesh sieve to catch any flukes or parts to flukes. The container is rinsed and this water also poured through the sieve.

h) The liver is placed back into the original container.

i) The liver is cut into segments approximately 20cm^3 to make slicing easier. Each segment is placed on the white tray for processing.

j) Liver segment examined for any flukes and these placed in the 90ml container.

k) Any large bile ducts cut using large scissors. These are often calcified so smaller scissors would not cut. If the bile ducts ran deep into the liver parenchyma, then cut down to this using the knife and continue to open up the bile ducts.

l) Once open remove any flukes evident. Flush with water bottle to remove and flukes and fragments.

m) When liquid had collected in the white tray, pour this into the sieve before any was lost.

n) Slice the liver into 1-2cm slices and examine each. If bile ducts became evident then incise these open with scissors.

o) Once 10-15 slices had been made cut that section of liver off and put it into the first 10 litre bucket. Rinse under running water massaging the slices to remove any flukes or segments.

Place rinsed liver into the second bucket (for eventual disposal). After rinsing 3-4 segments pour the liquid in the bucket into the sieve. This would also contain liver fragments

p) Continue this process until the entire liver has processed; bile ducts opened and flushed, liver parenchyma sliced, rinsed, rinse water sieved, liver placed in second bucket.

q) Rinse the white tray into the larger container that the liver had been stored in, and then rinse this through the sieve.

r) If large liver particles are in the sieve, rinse these under running water to remove any fluke particles and discard liver.

s) Upend sieve onto white tray. Bang it onto the tray to remove heavier materials. Use the 1 litre bottle to flush the sieve, turning it to aid removal of all the material onto the white tray. Tap the sieve heavily onto the ball of the hand to help remove all fragments. Visually check that all are removed.

t) Ensuring good light and using a magnifying lens if required remove all flukes, segments and suspicious materials into the 90ml pottle. Examine the tray left to right, back again and repeat this process so that the tray is examined 4 times.

u) Pour contents of 90ml container into dishes and examine under the dissecting microscope.

v) Remove entire flukes and place on a second wetted disk (to prevent them adhering).

w) Place fluke segments on a separate part of the dish, discard liver fragments.

x) Count flukes and segments.

y) Segments; if >75% then = 1 fluke

    i. Oral sucker present = 1 fluke

3.1.5 Standard Operating Procedure 5: In-House serum antibody ELISA protocol

Purpose

Serum antibody ELISA using Excretory – Secretory antigens as a diagnostic tool to identify *Fasciola hepatica* infection.

Materials and Equipment

- Grenier bio-one 96 well, flat, microlon high binding plates (reference 655061) Germany
- ES antigen as described in Section 3.1.3
- Positive control serum from naturally infected animal and assessed in a commercial laboratory using the IDEXX Fasciolosis Verification test.
- Negative control serum from a farm with no known liver fluke infection and assessed in a commercial laboratory using the IDEXX Fasciolosis Verification test.
- Bicarbonate buffer 0.05M
- Paraffin film
- PBS
- Distilled water for dilution
- Tween
- Skim milk powder (Countdown brand)
- TMB ultrafast solution
- Bovine IgG goat polyclonal, Bethyl Laboratories Inc. catalogue A10-130P
- Pipettes and tips
- Eppendorf 1.5ml
- 1N H_2SO_4 stop solution
- Plate reader 450 nm
- Sterile bottles for mixing reagents

Reagents and reagent preparation

The experimental protocol is based on the methodology from Salimi-Bejestani (Salimi-Bejestani *et al.* 2005)

a) ES antigen

Use 2ug/ml

Require 100ul/well of 2ug/ml ES antigen in 0.05M HCO3

1660ug/ml X = 2ug/ml x 10,000 ul

X = (2 x 10,000) / 1660

X = 20,000 / 1660

X = 12.05 ul

Add 12.05ul ES to (10,000 – 12.05) = 9988ul of 0.05M HCO3 buffer

At 100ul per well this give sufficient ES for 100 wells which gives POS control and NEG control duplicated plus 46 duplicate samples

b) Antigen preparation

- Remove ES extract from freezer
- Pipette bicarbonate buffer into mixing bottle
- Add ES at 12.05micolitre (ul) per 10,000 ml buffer
- Agitate
- Microlon hi binding flat bottom plate (Griener Bio-One ref 655061 Germany)

- 100 ul per well
- Cover with paraffin film
- Refrigerate overnight

c) PBS

Using PBS 10x dilute this down to x

Add 9 parts of distilled water (dH2O) to 1 part PBS 10x

This product has a long storage life so make up 1000ml by mixing 100ml PBS 10x to 900ml dH2O

Volume required per plate;

3 wash cycles comprising 3 + 2 + 3 + 2 + 3 + 2

15 washes @300ul/well using PBS / Tween 0.05%

One well = 96 plates

96 x 15 x 300ul = 432,000ul

Plus

To make up Blotto @ 200ul/well

Blotto = PBS + Tween + skim milk powder

200ul/well x 96 wells = 19,200ml

Plus

To make up Blotto for each sera @800ul

48 x 800 = 38,400ul

Total PBS = 432,000 + 19,200+ 38,400 = 489,600 ul or 489ml so make up 500ml

So add 50ml PBS 10x to 450ml dH2O.

d) Wash Fluid PBS / Tween 0.05% (PBS/T)

To make up 500ml wash solution add 250ul Tween 0.5% to 500ml PBS

Agitate gently

e) Blotto 5%

skim milk powder (SMP) added to PBS/Tween mix

Require 200ul/well; 200ul x 96 = 19,200ul

10000ul for secondary

38,400 for 48 serums

= 67,600ul

so make 100,000ul or 100ml per plate

100 x 2% = 100 x .02 = 5g SMP to 100ml PBS/T

This will keep overnight when refrigerated.

f) TMB ultrafast solution

100ul per well warmed to room temperature = **9600ul.**

g) Secondary Block

Bethyl goat Anti-bovine @ 1:60,000 mix with Blotto

1ul secondary to 60,000ul Blotto

100ul/well =9600ul

h) Serum

100ul per well so 200ul per sample

1:800 dilution in Blotto, so require 800ul Blotto per sample as this will make 800ul of sample which is ample

Add 800ul Blotto to 1.5ml Eppendorf

Add 1ul serum

Agitate

Agitate again before pipetting onto plate

96 well = 48 serums duplicated = 48 x 800ul = 38,400ul Blotto required

Mix 1ul serum to 800ul Blotto

i) Positive Serum : #347 from known infected farm (strong positive on IDEXX, SP% 244%
j) Negative Serum : N2 (from farm with no history of liver fluke and IDEXX SP% 2% and 4%)
k) Stop Solution

1N H_2SO_4 100ul/well **=** 9600ul

l) Read at 450nm

Calculation

m) Optical Density Ratio (ODR) calculation

C+ optical density (OD) = Average C+ (OD)

C- OD = average C- OD

Sample OD = average sample OD

Modified OD C+ = C+ OD minus C- OD

Modified OD sample = sample OD minus C- OD

ODR = (modified sample OD)/ (modified C+ OD)

Procedure

n) Microlon high binding plate
o) 100ul/well of ES @ 2ug/ml
p) Cover with paraffin film and incubate refrigerated overnight

Next day

q) Remove paraffin film
r) Wash 300ul/well 2 short washes
s) Block with 200ul Blotto per well
t) Cover with plastic lid, incubate 37C for 60 minutes
u) Place TMB in incubator to warm to room temperature
v) Tap out contents
w) Add 100ul per well of duplicated serum
x) Cover with plastic lid, incubate 37C for 60 minutes
y) Wash 300ul/well 3 short washes, option 6 automatic washer
z) Wash 300ul/well 2 long washes (5 minutes each), option 17 automatic washer
aa) Add 100ul per well of 1:60,000 secondary
bb) Cover with plastic lid, incubate 37C for 60 minutes
cc) Remove TMB from incubator
dd) Wash 300ul/well 3 short washes, option 6 automatic washer
ee) Wash 300ul/well 2 long washes, option 17 automatic washer
ff) Add 100ul TMB at room temperature
gg) Agitate slowly, incubate 37C covered for 10 minutes agitating occasionally or until colour reaction is sufficient
hh) Add 100ul/well stop solution
ii) Read at 450nm.

3.1.6 Standard Operating Procedure 6: Coproantigen ELISA protocol

Purpose

Faecal antigen ELISA as a diagnostic tool to qualify and quantify active *Fasciola hepatica* infection.

Materials and Equipment

1) Faecal samples collected, chilled or frozen at -20C for up to 795 days.
2) Pipettes and tips
3) Sterile bottles for mixing reagents
4) Distilled water

5) 10ml plastic red top vacutainer or similar 1ml screw top plastic measuring tube (to hold faecal sample and diluent)
6) Commercial test kit: BIO K 201 - Monoscreen AgELISA Fasciola hepatica
   Bio-X Diagnostics S.A.
   Rue de la Calestienne, 38 (PAE)
   5580 ROCHEFORT
   Belgium
7) Plate reader 450nm

Procedure

The procedure was altered compared to the manufacturer's instructions (Brockwell *et al.* 2013) to include an overnight incubation of the faeces in the diluent and the use of a lower cut-off value of 0.014.

a) Bring all the reagents at 21°C +/- 3°C before use.
b) Mix dilution buffer; 100ml 5X buffer concentrate to 450ml distilled water.
c) Dilute the faecal material in the dilution buffer (2 g + 2 ml for cattle and 0.5 g + 2 ml for ovine). Store overnight on the laboratory bench.

Next day

d) Remove the microplate from its packaging.
e) Invert faecal material and diluent to mix. Centrifuge 10 min. at 1,000 g. Collect supernatants.
f) Add 100-µl aliquots of the diluted samples to the wells as follows: sample 1 in wells A1 and B1, sample 2 in wells C1 and D1, etc. Proceed in the same manner for the positive reference (example: G1 and H1).
g) Cover the plate with a lid and incubate at 21°C +/- 3°C for 2 hours on a plate agitator.
h) Prepare the washing solution as instructed in the section "Composition of the Kit". Add 100ml 20X wash concentrate to 1900ml distilled water.
i) Wash three times in automatic washer, 300 µl of the washing solution per well.
j) Dilute the necessary amount of the biotin-linked anti-Fasciola hepatica conjugate fiftyfold in the reagent dilution buffer (20 µl of conjugate + 980 µl of the reagent dilution buffer per strip).
k) Add 100 µl of the diluted anti-Fasciola hepatica conjugate solution to each well.
l) Cover with a lid and incubate the plate at 21°± 3°C for one hour.
m) Wash three times in automatic washer, 300 µl of the washing solution per well.
n) The avidine-peroxidase conjugate is liquid and must be diluted fiftyfold in the reagent dilution buffer (20 µl of conjugate + 980 µl of the reagent dilution buffer per strip).
o) Add 100 µl of the diluted peroxidase-linked conjugate solution to each well.
p) Cover with a lid and incubate the plate at 21°± 3°C for one hour.
q) Wash three times in automatic washer, 300 µl of the washing solution per well.
r) Add 100 µl of the chromogen solution to each well on the plate. The chromogen solution must be absolutely colourless when it is pipetted into the wells. If a blue colour is visible, this means that the solution in the pipette has been contaminated.

s) Incubate at 21°C +/- 3°C and away from light for 10 minutes. Do not cover. This time interval is given as a guideline only, for in some circumstances it may be useful to lengthen or shorten the incubation time.
t) Add 50 µl of stop solution per microwell. The blue colour will change into a yellow colour.
u) Read the optical densities in the microwells using a plate reader and a 450 nm filter. Results must be read fairly soon after the stopping solution has been added since the chromogen may crystallize in wells with strong signals and distort the results accordingly

Calculating the "Value"

v) Calculate the net optical density of each sample by subtracting from the reading for each sample well (A, C, E, G) the optical density of the corresponding negative control (B, D, F, H). Proceed in the same way for the positive control antigen.
w) The test is validated only if the positive control antigen yields a difference in the optical density at 10 minutes that is greater than the validation value given on the QC data sheet.
x) Divide the signal read for each sample well by the corresponding positive control signal and multiply this result by 100 to express it as a percentage.
y) Value = (Delta OD Sample * 100) / Delta OD Positive
z) A value >0.014 is positive for *Fasciola hepatica* infection.

3.1.7 Standard Operating Procedure 7: Serum collection, preparation and storage

Principle

To collect, prepare and store the serum from blood samples collected from animals.

Materials and Equipment

1) Vacutainers (10ml plastic, red top), numbered sequentially
2) Needle holders and needles
3) Sharps disposal containers
4) Disposable gloves
5) Black bin liners for rubbish
6) Portable table
7) Recording device (cell phone in top pocket is suitable)
8) Aerosol raddle marker
9) Disinfectant and protective clothing for entering farm and sampling animals
10) Chiller packs to accompany blood samples send to laboratory
11) Centrifuge
12) Eppendorf tubes, 0.8 – 1.6ml, two per sample
13) Labels for each eppendorf tube (two per sample) stating sample number, farm name, date of collection, one marked with R as reserve sample
14) Pipettes and tips
15) Freezer

Procedure

Before arrival on farm

a) Sequentially number vacutainers.
b) Assemble vacutainer holders and needles and place on trays.
c) If resampling cows, arrive the day before sampling and spray aerosol mark the cows for sampling the next day

On farm

d) On arrival at farm follow any biosecurity procedures required.
e) Set up equipment on portable table.
f) Stand on vet examination trolley to blood sample cows.
g) Blood sample cow from caudal vein and voice (paper record also if you have a helper) the cow tag number and vacutainer number.
h) Store blood sample upright in polystyrene holders they are purchased in or in plastic tray for sorting at end.
i) Place used needle and holder in plastic bucket to later be sorted with needles placed in sharps disposal container.
j) At the end of sampling sort all samples
k) Place vacutainer in chilly bins (if very cold when sampling (<15C), do not chill the vacutainers further as this prevents good clot formation making serum extraction more difficult later).
l) Clean all equipment.
m) Place rubbish in bin liner.
n) Help farmer clean shed.
o) Collect 70ml bulk milk sample from the vat and freeze.
p) Send chilled samples to Massey university on overnight courier.

Laboratory

q) Place milk sample in freezer
r) Place vacutainers in chiller
s) Centrifuge vacutainers at xx
t) Pipette serum into the two eppendorf tubes and label with the same number on the vacutainer.
u) Discard used pipette tips and vacutainer in biological waste.
v) Freeze serum sample at -20C (split the primary and reserve samples in case on freezer malfunctions).

3.1.8 Standard Operating Procedure 8: Bulk Milk Sample Collection and Storage

Principle

Collect bulk milk samples from herds at recorded interval for later analysis for the presence of *Fasciola hepatica* antibodies.

Materials and Methods

16) Milk sample pottles with a screw top lid, sequentially numbered and farm name.
17) Recording sheet for farmer.

Procedure

a) contact farmer to outline principle of the milk sampling and recording.
b) identify milk collection pottles for each farm and sequentially number.
c) Prepare recording sheet for each farm to identify the date of milk collection for each pottle.
c) post sample pottles, recording sheet and instruction to farmers.
d) farmers to collect milk from vat and record sample collection date.
e) store milk samples in freezer.
f) collect milk samples from farmer and transport to laboratory frozen.
g) store frozen milk samples.

3.1.9 Standard Operating Procedure 9: IDEXX Fasciola hepatica antibody test

Principle

The detection of antibodies specific to *Fasciola hepatica* in serum and milk and interpretation of this result to determine the severity of infection or prevalence of infection in the sampled group.

Materials and Equipment

1) Pipettes and tips
2) Sterile mixing bottles
3) Microtubes 1.1ml with caps
4) IDEXX Fasciolosis Verification, Fasciola hepatica antibody test kit, IDEXX Laboratories, Inc. One IDEXX Drive, Westborrk, Maine 04092, USA
5) Mixing tubes and caps
6) Vortex
7) Microplate washer
8) Deionised water
9) Incubator 37C
10) Plate reader 450nm.

Procedure

a) Thaw samples for analysis (serum or milk)
b) Bring reagents to room temperature
c) Create wash solution by diluting wash concentrate 20X with deionised water eg. 15ml of wash concentrate to 285ml deionised water, mix well are 18-26C. This is stable for 3 days when stored at 2-8C.
d) Dilute Conjugate 100X with Dilution Buffer N.1 eg. 10ul Conjugate concentrate with 990ul Dilution Buffer N.1. Mix well. This solution is stable for 8 hours at 18-26C.
e) Centrifuge milk samples at 1000g for 5 minutes.
f) Record the sample identification for referencing to the plate reader results.

Mixing tube preparation serum samples

g) Load mixing tubes into holders. These will hold the dilution buffer and sample for each plate. Once all serum or milk samples are loaded into the mixing tubes, vortex them and pipette onto plates
h) Dispense 380ul of Dilution Buffer N.2 into each mixing tube
i) Dispense 20ul negative control into first tube
j) Dispense 20ul positive control into the next two tubes
k) Dispense 20ul of each serum sample into the mixing tubes in the sequence below

C-					
C+	Sample #7				
C+	continuation				
Sample #1					
Sample #2					
Sample #3					
Sample #4					
Sample #5					

Mixing tube preparation milk samples

a) Dispense 380ul of Dilution Buffer N.2 into the first there mixing tubes in column 1
b) Dispense 20ul negative control into first tube
c) Dispense 20ul positive control into the next two tubes
d) Dispense 200ul of skim milk into the mixing tubes as for the serum samples

e) Vortex samples
f) Transfer 200ul of each sample into the corresponding wells of the plates from the test kit in the sequence bellow using a multichannel pipette

	1	2	3	4	5	6	7	8	9	10	11	12
A	C-	C-	#6	#6								
B	C+	C+										
C	C+	C+										
D	#1	#1										
E	#2	#2										
F	#3	#3										
G	#4	#4										
H	#5	#5										

g) Gently agitate plate.
h) Incubate covered, 37C, 60 minutes.
i) Automatic washer, 300ul/ well, 3 short wash and two long 5 minute washes.
j) Dispense 100ul diluted conjugate into each well.
k) Incubate covered, 37C, 30 minutes.
l) Automatic washer, 300ul/ well, 3 short wash and two long 5 minute washes.
m) Dispense 100ul TNMB substrate N.12 into each well.
n) Incubate 20 minutes (or sooner depending on rapidity of colour change) at rom temperate away from direct sunlight. The empty testing kit box was useful for this.
o) Dispense 100ul Stop Solution N.3 inot each well
p) Read plate at 450nm

q) Record optical densities
r) Calculate SP% as per kit instructions
s) Interpret SP% to diagnostic category as per IDEXX manual

		IDEXX ELISA Interpretation	
SP%		**Prevalence of Infestation (pooled samples)**	**Infection description (individual samples)**
≤30	0	Negative	no or very weak
30.1-80	+	<20%	low
80.1-150	++	20-50%	medium
≥150	+++	>50%	strong (high)

t) Tabulate results to individual cow identification of bulk milk sample

3.1.10 Standard Operating Procedure 10: Liver Scoring Protocol (from Sargent et al 2009)

Liver Score	Pathology description
0	Absolutely no pathology evident – liver normal colour, consistency and no visible sign of fluke lesions
1	Small areas of scar tissue and lesions, <5% of liver affected
2	Moderate areas of scar tissue and lesions present, occurring in 5-10% of the liver
3	Moderate areas of scar tissue, thickening of the bile ducts, small to moderate areas of necrosis, pus, 10-20% of liver affected
4	Moderate to large areas of scar tissue, “pipe stem” liver starting to occur. Moderate areas of necrosis pus, haemorrhage, 20-30% of liver affected
5	Large areas of scar tissue, and pipe stem liver. Multiple necrotic foci, pus, haemorrhage, severe degeneration and >30% of liver affected

3.2 Supplementary Materials

3.2.1 Chapter 2 cattle data

Cow	age	Total fluke Count	Liver score	Fluke Eggs / 10g faeces	epg	Serum IH ELISA	IH descriptive	Coproantigen Value	Copro description	IDEXX SP%	IDEXX description	Liver weight kg	liver % liveweight	live wt kg	carcass weight (kg)	yield
1	MAC	11	5	0	0	2.71	positive	0.528052805	negative	272.4489796	strong	8.09	1.47%	548.7	258	47%
2	MAC	15	5	1	0.1	0.79	positive	27.06270627	positive	234.6938776	strong	8.88	1.47%	605.8	295	49%
3	MAC	57	5	0	0	1.34	positive	33.26732673	positive	248.3673469	strong	8.32	1.78%	467.1	218	47%
4	MAC	3	5	2	0.2	2.83	positive	12.80528053	positive	244.0816327	strong	7.05	1.44%	488.5		
5	MAC	9	5	11	1.1	2.54	positive	51.68316832	positive	342.8571429	strong	6.67	1.42%	468.1	218	47%
6	MAC	10	5	7	0.7	1.76	positive	18.94389439	positive	276.9387755	strong	8.32	1.57%	528.3	230	44%
7	MAC	32	5	11	1.1	1.78	positive	55.37953795	positive	288.1632653	strong	7.4	1.38%	536.5	247.5	46%
8	MAC	8	5	0	0	1.56	positive	27.12871287	positive	129.1836735	positive	8.33	1.57%	530.4	240	45%
9	MAC	0	5	0	0	1.3	positive	-0.330033003	negative	173.4693878	strong	8.3	1.40%	593.6	280	47%
10	MAC	26	5	4	0.4	1.95	positive	55.37953795	positive	154.6938776	strong	7.69	1.60%	481.1	206.5	43%
11	MAC	12	5	7	0.7	2.07	positive	32.60726073	positive	191.0204082	strong	8.28	1.56%	531.4	245	46%
12	MAC	5	5	9	0.9	1.19	positive	4.620462046	positive	188.9795918	strong	7.26	1.35%	538.5	257.5	48%
13	MAC	51	5	20	2	1.83	positive	44.29042904	positive	290.8163265	strong	7.24	1.49%	487.5	212	43%
14	MAC	6	5	3	0.3	1.53	positive	-2.112211221	negative	253.4693878	strong	6.5	1.31%	497.7	239	48%
15	MAC	8	5	6	0.6	2.34	positive	13.86138614	positive	251.2244898	strong	7.63	1.46%	523.2	220	42%
16	MAC	7	5	1	0.1	2.19	positive	9.438943894	positive	250.8163265	strong	7.24	1.45%	498.7		
17	MAC	7	5	0	0	1.32	positive	-0.066006601	negative	272.8571429	strong	7.12	1.28%	555.9	292.5	53%
18	MAC	3	5	1	0.1	1.69	positive	4.554455446	positive	223.0612245	strong	8.35	1.47%	569.1	258.5	45%
19	MAC	6	5	11	1.1	1.54	positive	5.280528053	positive	184.6938776	strong	6.79	1.44%	472.2	208.5	44%
20	MAC	74	5	35	3.5	1.94	positive	58.74587459	positive	167.9591837	strong	8.91	1.65%	541.6	235	43%
21	MAC	4	5	1	0.1	1.99	positive	23.03630363	positive	212.4489796	strong	7.75	1.56%	495.7	245.5	50%
22	MAC	14	4	9	0.9	2.12	positive	16.69966997	positive	242.8571429	strong	7.29	1.33%	549.7	280.5	51%
23	MAC	7	5	0	0	2.81	positive	22.83828383	positive	286.7346939	strong	7.16	1.56%	457.9	215.5	47%
24	MAC	4	5	1	0.1	1.62	positive	2.04620462	positive	264.2857143	strong	6.87	1.34%	512	233.5	46%
25	MAC	8	5	1	0.1	1.1	positive	2.640264026	positive	165.5102041	strong	7.27	1.47%	494.7	236.5	48%
26	MAC	7	5	0	0	2.31	positive	12.47524752	positive	215.3061224	strong	7.79	1.49%	522.2	247	47%
27	MAC	1	5	0	0	1.7	positive	1.188118812	negative	297.9591837	strong	7.92	1.32%	599.7		
28	MAC	9	4	9	0.9	1.69	positive	19.27392739	positive	271.2244898	strong	6.64	1.60%	416.1	199.5	48%
29	MAC	4	5	0	0	1.97	positive	2.574257426	positive	204.6938776	strong	8.09	1.46%	554.8	244.5	44%
30	R2	10	4	3	0.3	2.59	positive	12.54125413	positive	290.8163265	strong	6.6	1.28%	517.1	256.5	50%
31	R2	3	4	11	1.1	1.94	positive	8.712871287	positive	279.1836735	strong	7.26	1.11%	656.8	350	53%
32	R2	4	4	4	0.4	2.55	positive	0	negative	251.0204082	strong	7.35	1.10%	666	342.5	51%
33	R2	0	4	3	0.3	1.82	positive	54.52145215	positive	271.8367347	strong	7.49	1.11%	676.2	358.5	53%
34	R2	4	4	52	5.2	1.88	positive	-0.066006601	negative	262.244898	strong	9.12	1.74%	524.2	285.5	54%
35	R2	3	4	6	0.6	2.07	positive	-0.132013201	negative	303.0612245	strong	8.09	1.31%	618.7	317.5	51%
36	R2	0	3	6	0.6	2.6	positive	2.244224422	positive	300.4081633	strong	6.37	0.93%	686.4	380.5	55%
37	R2	7	2	1	0.1	1.91	positive	1.386138614	negative	276.122449	strong	7.65	1.24%	619.1	318	51%
38	R2	0	4	11	1.1	1.9	positive	0.924092409	negative	305.3061224	strong	7.6	1.16%	655.8	361.5	55%
39	R2	0	5	1	0.1	1.59	positive	27.98679868	positive	284.8979592	strong	8.43	1.44%	583.4	280	48%

3.2.2 Chapter 3 farmer survey questionnaire and responses

Please take the time to tick the boxes to the questions concerning liver fluke on your farm.

Westland Supplier ____________________

1. Are you aware of liver fluke being present on your farm at any time in the past 5 years?

Yes	No

2. Did you drench the cows with a product that specifically targets liver fluke?

Yes	No

3. If you answered **yes** to the previous question, what portion of the herd did you treat

less than 25%	25-75%	more than75%	100%

4. Did you drench other ages of cattle with a drench that specifically targets liver fluke?

Calves (R1)	Yes	No
Heifers (R2)	Yes	No

5. Are you interested in attending a workshop / seminar on liver fluke?

Yes	No

6. We are interested in studying the effects that liver flukes have on milk production in 5 herds. If you interested in being part of these studies, provide you most convenient contact details

Name____

Email____

Phone____

farm id	region	IDEXX SP% March	IDEXX SP% October	1 yes	1 no	1 no answer	2 yes	2 no	3 <25%	3 25-75%	3 >75%	3 100%	4 R1 drench yes	4 R1 drench no	no answer R1 drench	4 R2 drench yes	4 R2 drench no	no answer R2 drench
1	Fox/Franz	1.9	5		1			1						1			1	
2	Fox/Franz	3.6	14	1				1					1			1		
3	Fox/Franz	27.7	15	1				1					1			1		
4	Whataroa/Te Taho	39.1	97	1			1					1	1			1		
5	Whataroa/Te Taho	164.4	239	1			1				1		1			1		
6	Whataroa/Te Taho	236.5	250	1			1					1	1			1		
7	Whataroa/Te Taho	194.7	216	1			1					1	1			1		
8	Whataroa/Te Taho	156.4	173	1			1					1	1			1		
9	Whataroa/Te Taho	203.6	117	1				1					1			1		
10	Whataroa/Te Taho	216.2	148	1				1					1			1		
11	Whataroa/Te Taho	210.5	74	1				1					1			1		
12	Whataroa/Te Taho	205	146	1				1					1			1		
13	Whataroa/Te Taho	152.9	119	1				1					1			1		
14	Whataroa/Te Taho	182.9	94	1				1					1			1		
15	Whataroa/Te Taho	246.7	284	1				1						1			1	
16	Whataroa/Te Taho	186.9	150		1		1					1	1			1		
17	Whataroa/Te Taho	176.5	34	1			1					1	1			1		
18	HariHari/Evans Creek	102.4	31	1			1					1	1			1		
19	HariHari/Evans Creek	7	56	1			1					1	1			1		
20	HariHari/Evans Creek	42.9	89	1			1					1	1			1		
21	HariHari/Evans Creek	87.6	176	1			1					1	1			1		
22	HariHari/Evans Creek	233.1	221	1			1					1	1			1		
23	HariHari/Evans Creek	214.8	160	1			1					1	1			1		
24	HariHari/Evans Creek	250.5	143	1			1					1	1			1		
25	HariHari/Evans Creek	234.7	115	1			1					1	1			1		
26	HariHari/Evans Creek	91.6	24	1			1					1	1			1		
27	HariHari/Evans Creek	139.3	92	1			1					1	1			1		
28	HariHari/Evans Creek	161.3	94	1			1					1			1	1		
29	HariHari/Evans Creek	226.4	236	1			1					1	1			1		
30	HariHari/Evans Creek	271.3	206	1			1					1	1			1		
31	HariHari/Evans Creek	175.8	244	1			1					1	1			1		
32	HariHari/Evans Creek	263	140	1			1					1	1			1		
33	HariHari/Evans Creek	220.2	239	1			1					1		1			1	
34	HariHari/Evans Creek	223.3	166	1			1					1	1			1		
35	Waitaha/Bold Head	106.4	110	1			1					1		1			1	
36	Waitaha/Bold Head	42.2	19	1			1					1	1				1	
37	Waitaha/Bold Head	204.6	162	1			1					1	1			1		
38	Waitaha/Bold Head	158.8	178	1			1					1	1			1		
39	Waitaha/Bold Head	106.2	26	1			1					1		1		1		
40	Ross	213.7	250	1			1					1	1			1		
41	Kokotahi/Kowhitirangi	193.1	180	1			1					1			1	1		
42	Kokotahi/Kowhitirangi	168.9	155	1			1					1			1	1		
43	Kokotahi/Kowhitirangi	176.3	93	1			1					1	1			1		
44	Kokotahi/Kowhitirangi	63.8		1			1					1	1			1		
45	Kokotahi/Kowhitirangi	236.3	242	1			1					1	1			1		
46	Kokotahi/Kowhitirangi	232.9	232	1			1					1	1			1		
47	Kokotahi/Kowhitirangi	230.9	214	1			1					1		1		1		
48	Kokotahi/Kowhitirangi	90.4	102	1			1					1	1			1		
49	Kokotahi/Kowhitirangi	166.6	193	1			1					1		1		1		
50	Kokotahi/Kowhitirangi	150.6	209	1			1					1		1			1	
51	Kokotahi/Kowhitirangi	193.8	158	1			1					1	1			1		
52	Kokotahi/Kowhitirangi	2.5	40			1	1					1	1			1		
53	Kokotahi/Kowhitirangi	187.7	107	1			1					1		1		1		
54	Kokotahi/Kowhitirangi	148.1	12	1			1					1		1		1		
55	Kokotahi/Kowhitirangi	224.2	50	1			1					1	1			1		
56	Kokotahi/Kowhitirangi	255.5	203	1				1					1			1		
57	Kokotahi/Kowhitirangi	80.3	130	1			1			1			1			1		
58	Kokotahi/Kowhitirangi	187	43	1			1					1			1	1		
59	Kokotahi/Kowhitirangi	177.1		1				1							1			1
60	Kokotahi/Kowhitirangi	38.7	32	1			1		1				1			1		
61	Kokotahi/Kowhitirangi	192.1	40	1			1					1	1			1		
62	Kokotahi/Kowhitirangi	255		1			1					1	1			1		
63	Kokotahi/Kowhitirangi	236.2	102	1			1					1	1			1		
64	Kokotahi/Kowhitirangi	234.5		1			1					1	1			1		
65	Arahura/Chesterfield	207.7	190	1			1					1		1		1		
66	Arahura/Chesterfield	238.1	102	1			1					1	1			1		
67	Arahura/Chesterfield	230.4	246	1			1					1	1			1		
68	Arahura/Chesterfield	188.7	147	1			1					1	1			1		
69	Arahura/Chesterfield	190.1	126	1			1					1		1			1	
70	Kumara/Taramakau	94.5	6	1			1					1	1			1		
71	Kumara/Taramakau	12.5	6		1			1					1			1		
72	Kumara/Taramakau	144.9		1			1					1	1			1		
73	Kumara/Taramakau	65.2	2	1			1					1			1			1
74	Kumara/Taramakau	4.2	3	1			1					1	1			1		
75	Kumara/Taramakau	61.8	39	1			1					1	1			1		
76	Kumara/Taramakau	9.8	19	1				1						1			1	
77	Inchbonne/Rotomanu	11.5	7	1			1					1	1			1		
78	Inchbonne/Rotomanu	70	25	1			1					1	1			1		
79	Inchbonne/Rotomanu	80.2	8	1			1					1	1			1		
80	Inchbonne/Rotomanu	87.2	68	1			1					1	1			1		

81	Inchbonne/Rotomanu	6.3	6	1			1					1	1			1		
82	Inchbonne/Rotomanu	6.6	3	1				1						1			1	
83	Inchbonne/Rotomanu	256.9	123	1			1					1	1			1		
84	Hbell Hill/Haupiri/Nelso	215.2		1			1					1	1			1		
85	Hbell Hill/Haupiri/Nelso	43.5	22	1				1						1			1	
86	Hbell Hill/Haupiri/Nelso	61.7	9		1			1						1			1	
87		57.6	28	1			1		1				1				1	
88	Greymouth to Reefton	205.6	36	1			1					1	1			1		
89	Greymouth to Reefton	138.2	63	1			1					1	1				1	
90	Greymouth to Reefton	11.6	3	1				1						1			1	
91	Greymouth to Reefton	101.1	37	1			1					1	1			1		
92	Greymouth to Reefton	3.4	5		1			1						1			1	
93	Greymouth to Reefton	2.8	2		1			1					1			1		
94	Greymouth to Reefton	60.2	33	1			1				1			1			1	
95	Greymouth to Reefton	19.5	20	1				1						1		1		
96	Greymouth to Reefton	23.5	32		1			1						1			1	
97	Greymouth to Reefton	46.8	48	1			1					1	1			1		
98	Greymouth to Reefton	3.8	3	1				1						1			1	
99	Greymouth to Reefton	1.7	0		1			1						1			1	
100	Greymouth to Reefton	1.1	4		1			1						1			1	
101	Greymouth to Reefton	31.7	35	1			1					1	1			1		
102	Greymouth to Reefton	6.7	2	1			1					1	1			1		
103	Greymouth to Reefton	21.2	64		1			1						1			1	
104	Greymouth to Reefton	80.1	13	1			1					1		1			1	
105	Greymouth to Reefton	274.2	254	1			1					1		1			1	
106	Greymouth to Reefton	3.7	132	1			1					1		1			1	
107	Greymouth to Reefton	53.3	17	1			1					1	1			1		
108	Greymouth to Reefton	174.9	44	1			1					1	1			1		
109	Greymouth to Reefton	42.4	49	1			1					1		1			1	
110	Greymouth to Reefton	4.6	9	1			1					1		1			1	
111	Greymouth to Reefton	4.4		1				1					1					1
112	Greymouth to Reefton	85		1			1					1		1			1	
113	Maruia/Springs Junctio	1.4	1		1			1						1			1	
114	Reefton	14.8	6	1			1					1	1			1		
115	Reefton	40.8	10	1			1					1	1			1		
116	Reefton	0.6	7		1			1					1			1		
117	Reefton	5.4	0	1				1						1			1	
118	Reefton	4.1	12	1			1					1	1			1		
119	Reefton	52.5	5		1			1						1			1	
120	Reefton	16.7	12		1		1					1	1			1		
121	Inangahua/Shanandoah	9.6	3	1			1		1				1			1		
122	Inangahua/Shanandoah	134.9	127	1				1					1					1
123	Inangahua/Shanandoah	0.4	0	1				1						1			1	
124	Inangahua/Shanandoah	18.4	19	1				1						1			1	
125	Barrytown	203.8	109	1			1					1	1			1		
126	Barrytown	191.1	90	1			1					1			1		1	
127	Barrytown	145.7	15		1			1						1			1	
128	Westport to Seddon	206.4	129	1			1					1	1					1
129	Westport to Seddon	196.8	103	1			1					1	1			1		
130	Westport to Seddon	220.2	87	1			1					1	1			1		
131	Westport to Seddon	174	89	1			1					1	1			1		
132	Westport to Seddon	7.7	6	1			1					1	1			1		
133	Westport to Seddon	156.8		1			1					1		1		1		
134	Westport to Seddon	152.6	17	1			1					1	1			1		
135	Westport to Seddon	209.7	202	1			1					1	1			1		
136	Westport to Seddon	256.3	199	1				1						1			1	
137	Westport to Seddon	30.1	21	1			1					1	1			1		
138	Westport to Seddon	240.6	241	1			1					1	1			1		
139	Westport to Seddon	63.5	37	1			1					1	1			1		
140	Westport to Seddon	148.2	75	1			1					1	1			1		
141	Westport to Seddon	69.8	94	1			1					1		1			1	
142	Karamea	238.5	105	1			1					1	1			1		
143	Karamea	233.5		1			1					1	1			1		
144	Karamea	233.1	151	1			1					1	1			1		
145	Karamea	249.5	153	1			1					1	1			1		
146	Karamea	110.1	55	1				1						1			1	
147	Karamea	235	164	1			1					1	1			1		
148	Karamea	92.9	33	1			1					1	1			1		
149	Karamea	251.1	225	1			1					1	1			1		
150	Karamea	3.2	2		1			1						1			1	
151	Karamea	4.1	1	1				1					1				1	
152	Karamea	162.6	42		1			1						1			1	
153	Karamea	213.1	176	1				1					1			1		
154	Karamea	225.2	232	1			1					1	1			1		
155	Karamea	172	249		1			1						1			1	
156	Karamea	68	48		1			1						1			1	
157	Karamea	239.1	253		1			1					1			1		
158	Canterbury	2.1	3		1			1						1			1	
159	Canterbury	3.3			1			1						1			1	
160	Canterbury	3.4	9		1			1						1			1	
161	Canterbury	4.1	1		1			1					1			1		

3.2.3 Chapter 3 bulk milk ELISA results March and October

Results are held in Electronic Appendix. Supplementary files; *Chapter 3 BME results March and October*.

3.2.4 Chapter 4 electronic files

Supplementary files are held in Electronic Appendix, Supplementary files;

Chapter 4. Cross Sectional dataset Coproantigen and Milk

Chapter 4. Cross Sectional dataset IDEXX and Milk

Chapter 4 Supplementary figures and tables.

3.2.5 Chapter 5 electronic files

Supplementary files are held in Electronic Appendix, Supplementary files;

Chapter 5. Longitudinal dataset

3.2.6 Chapter 6 models

```
library(rjags)

library(runjags)

library(coda)

library(superdiag)

library(prevalence)

Model1

t1 = c(10, 44, 6, 7, 9, 11, 0, 22)

n1=109

t2 = c(8, 46, 0, 0, 34, 10, 0, 1)

n2=99

inits1 <- list(se1=0.05, se2=0.05,se3=0.05, pi1=0.7, pi2=0.7,sp1=0.05, sp2=0.05, sp3=0.05)

inits2 <- list(se1=0.95, se2=0.95,se3=0.95, pi1=0.1, pi2=0.1,sp1=0.95, sp2=0.95, sp2=0.95)

model1 <- "

model{

##herd A

p1[1] <- pi1*se1*se2*se3 + (1-pi1)*(1-sp1)*(1-sp2)*(1-sp3) ###111

p1[2] <- pi1*se1*(1-se2)*(1-se3) + (1-pi1)*(1-sp1)*sp2*sp3 ###100
```

```
p1[3] <- pi1*(1-se1)*se2*(1-se3) + (1-pi1)*sp1*(1-sp2)*sp3 ###010
 p1[4] <- pi1*(1-se1)*(1-se2)*se3 + (1-pi1)*sp1*sp2*(1-sp3) ##001
p1[5] <- pi1*se1*se2*(1-se3) + (1-pi1)*(1-sp1)*(1-sp2)*sp3 ###110
p1[6] <- pi1*se1*(1-se2)*se3 + (1-pi1)*(1-sp1)*sp2*(1-sp3) ##101
p1[7] <- pi1*(1-se1)*se2*se3 + (1-pi1)*sp1*(1-sp2)*(1-sp3) ###011
p1[8] <- pi1*(1-se1)*(1-se2)*(1-se3) + (1-pi1)*sp1*sp2*sp3 ###000
##herd B
p2[1] <- pi2*se1*se2*se3 + (1-pi2)*(1-sp1)*(1-sp2)*(1-sp3) ###111
p2[2] <- pi2*se1*(1-se2)*(1-se3) + (1-pi2)*(1-sp1)*sp2*sp3 ###100
p2[3] <- pi2*(1-se1)*se2*(1-se3) + (1-pi2)*sp1*(1-sp2)*sp3 ###010
 p2[4] <- pi2*(1-se1)*(1-se2)*se3 + (1-pi2)*sp1*sp2*(1-sp3) ##001
 p2[5] <- pi2*se1*se2*(1-se3) + (1-pi2)*(1-sp1)*(1-sp2)*sp3 ###110
p2[6] <- pi2*se1*(1-se2)*se3 + (1-pi2)*(1-sp1)*sp2*(1-sp3) ##101
p2[7] <- pi2*(1-se1)*se2*se3 + (1-pi2)*sp1*(1-sp2)*(1-sp3) ###011
p2[8] <- pi2*(1-se1)*(1-se2)*(1-se3) + (1-pi2)*sp1*sp2*sp3 ###000
###likelihood of contingency tables
t1[1:8] ~ dmulti(p1[1:8], n1)
t2[1:8] ~ dmulti(p2[1:8], n2)
 # Prior part:
 pi1 ~ dbeta(1, 1)
 pi2 ~ dbeta(1, 1)
 se1 ~ dbeta(23.6, 2.6)
 se2 ~ dbeta(24, 1.3)
 se3 ~ dbeta(6.4, 3.1)
 sp1 ~ dbeta(105.5, 3.8)
 sp2 ~ dbeta(24.4, 1.6)
 sp3 ~ dbeta(97, 1)
 # Hooks for automatic integration with R:
#data# t1, t2, n1, n2
#monitor# pi1, pi2, se1, se2, se3, sp1, sp2, sp3
}”
```

```
results <-run.jags(model1, adapt = 5000, n.chains=2, burnin=5000, sample=210000, inits=list(inits1,
inits2))
bayes.mod.fit.mcmc <- as.mcmc.list(results)
summary(bayes.mod.fit.mcmc)
superdiag(bayes.mod.fit.mcmc, burnin = 10000)
superdiagPlot(bayes.mod.fit.mcmc,burnin = 10000)
Model2
##get priors for coproantigen
betaExpert(best = 0.96, lower = 0.92, p = 0.95, method = "mean")
##model2
t = c(24, 28, 11, 22)
n=85
inits1 <- list(se1=0.05, se2=0.05, pi=0.7, sp1=0.05, sp2=0.05)
inits2 <- list(se1=0.95, se2=0.5, pi=0.1, sp1=0.95, sp2=0.95)
model2 <- "
model{
 # Likelihood part:
 p[1] <- pi*se1*se2 + (1-pi)*(1-sp1)*(1-sp2) ###11
 p[2] <- pi*se1*(1-se2) + (1-pi)*(1-sp1)*(sp2) ###10
 p[3] <- pi*(1-se1)*se2 + (1-pi)*(sp1)*(1-sp2) ###01
 p[4] <- pi*(1-se1)*(1-se2) + (1-pi)*(sp1)*(sp2) ###00
 # Prior part:
 pi ~ dbeta(1,1)
 se1 ~ dbeta(81, 3.4)
 se2 ~ dbeta(1, 1)
 sp1 ~ dbeta(81, 3.4)
 sp2 ~ dbeta(1, 1)
 ##likelihood of contingency tables
 t[1:4] ~ dmulti(p[1:4], n)
 # Hooks for automatic integration with R:
 #data# t, n
```

```
 #monitor# pi, se1, se2, sp1, sp2}"
results <-run.jags(model2, adapt = 5000, n.chains=2, burnin=5000, sample=210000, inits=list(inits1,
inits2))
bayes.mod.fit.mcmc <- as.mcmc.list(results)
summary(bayes.mod.fit.mcmc
```